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WOUND HEALING

ANNUAL PROGRESS REPORT

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Wound Healing; Regeneration; Liver Regeneration; Infection; Trauma; Injury; Burns; Fracture; Wound Infection; Vitamin A; Vitamin C; Thymosin; Adjuvants; Citral; DOCA; Metyrapone; Arginine; Tumor Angiogenesis Factor; Collagen; Cross-Linking; Fibroblasts; Fibroplasia; Tissue Culture; Environmental Temperature; Testosterone Propionate; Growth Hormone.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Studies of wound healing, regeneration and wound infection aimed at improving care of severely injured soldiers are underway in the following areas: (1) Adverse effects of severe injury on wound healing; attempts to improve healing in the severely injured. (a) Effects of injury on wound healing; (b) Additional studies dealing with attempts to increase fibroblastic proliferation, collagen synthesis, and/or collagen synthesis and thereby accelerate wound healing after injury. (2) Chemical accelerators of wound healing and regene-

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ration present in wounds and/or the blood. (a) Liver regeneration. (3) IV. Adverse effects of severe injury on resistance to infection; some aspects of wound infection and wound healing; attempts to increase local and systemic resistance to infection. (a) Thymosin; (b) Vitamin A, Metyrapone, and desoxycorticosterone.

Summary

Studies of wound healing, regeneration and wound infection aimed at improving care of severely injured soldiers are underway in the following areas:

II. Adverse effects of severe injury on wound healing; attempts to improve healing in the severely injured.

(1) Effects of injury on wound healing;

(2) Additional studies dealing with attempts to increase fibroblastic proliferation, collagen synthesis, and/or collagen synthesis and thereby accelerate wound healing after injury.

III. Chemical accelerators of wound healing and regeneration present in wounds and/or the blood.

(1) Liver regeneration

IV. Adverse effects of severe injury on resistance to infection; some aspects of wound infection and wound healing; attempts to increase local and systemic resistance to infection.

(1) *Thymosin;*

(2) Vitamin A, Metyrapone, and desoxycorticosterone.

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

WOUND HEALING

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I. Introduction

(a) General Aims

The reported studies are part of our continuing investigations supported by the Army Medical Research and Development Command aimed towards improving the wound healing of severely injured soldiers. Since the wounded man must heal his wounds to recover and return to duty, an understanding of the basic biochemical, physiologic and morphologic factors involved in healing and how these are affected by serious injury is fundamental to establishing improved prophylactic and therapeutic measures.

We have mentioned in our previous progress reports that wound healing may be considered as a specific biological process related to the general phenomenum of growth and regeneration. The various stages (including inflammation, blood vessel and lymphatic formation, fibroblastic proliferation, mucopolysaccharide, glycoprotein and collagen synthesis, collagen cross-linking, collagen resorption, remodelling of the wound, etc.) indicate that the entire process is an orderly one, showing a high degree of integration and organization characteristic of the processes in which control mechanisms are operative.

Our studies are based on our view that some of the key steps in these processes are affected adversely by injury and these may be modified in ways to improve the rate and quality of wound healing and thereby shorten convalescence. Since wound healing is an integrated process, factors which stimulate or inhibit one phase of the process have an effect on the overall process, the magnitude of the effect depending in part on how "rate-limiting" is the facet of healing being affected. These periods are not sharply separated. Included is a continuation of our studies of chemical accelerators of healing possibly present in wounds and/or the blood. Studies of liver regeneration are included because we believe that investigation of humoral factors which regulate and modulate liver regeneration are closely related to the question of why a wound begins to heal, what keeps it going, and what stops it. Also, liver injury severe enough to require hepatic resection is not rare in seriously injured soldiers. Since wound healing and wound infection are so closely interrelated, studies directed towards the prevention and control of wound infection and its local and systemic effects are included.

This progress report describes our studies dealing with:

I. Attempts to ameliorate the adverse effects of serious injury on wound healing by various nutritional, chemical, and hormonal means, singly and in combination, with emphasis on reparative vessel formation, fibroblastic proliferation, collagen synthesis, and collagen cross-linking.

II. Demonstration, isolation and chemical identification of possible humoral stimulators and regulators of wound repair and liver regeneration.

III. Attempts to improve by various chemical and hormonal means the depressed local and systemic resistance to infection associated with serious injury.

(b) Methods for Assessment of Wound Healing

Some of the ways in which we have assessed the effects of the various experimental regimens follow. Almost all of these have been used by us for a number of years and are well established in our laboratory.

Standard dorsal skin incisions are made and polyvinyl alcohol sponges of fixed size and weight implanted subcutaneously (1). In some experiments, mesh cylinders, stainless steel or nylon, are implanted (2). The wounds are closed with fine stainless steel sutures. All operations are conducted aseptically.

At various times postoperatively, the animals are killed and the wound and sponge granulomas excised. Using methods previously described by us (1, 3) (J. Trauma, 4: 543 (1964); Ann. Surg. 161: 293 (1965), the breaking strengths of adjacent wound strips are tested immediately or after fixation in buffered 10% formalin. We devised a new multiple guillotine so that the strips may be weighed. Other wound strips are examined histologically. The sponge granulomas are also examined histologically. In addition, hydroxyproline is measured as an index of reparative collagen. In some cases, the degree of cross-linking of the reparative collagen is estimated by the measurement of salt-soluble and salt-insoluble collagen. The amino acid composition of the newly formed collagen is determined in some experiments. Other measurements include the DNA and RNA contents of the granulomas and the uptake of ^{3}H-Thymidine into the DNA of the granulomas.

When stainless steel mesh cylinders are implanted, the wound fluids are analyzed for their chemical composition and the presence of wound-healing accelerating factor(s).

The methods for assessment may be tabulated as follows:

(1) General measurements

a) Body weight, food and water intakes (ad libitum and paired-feeding techniques), BUN, plasma proteins, Hct, WBC, differential, and various other chemical measurements in tissues, blood and urine as required, e.g., vitamin A.

b) Metabolic balance studies (food, urine, feces).

(2) Assessment of fibroblasts - number and appearance

a) Histologic appearance of incisions and sponge and cylinder regenerative tissues (light microscopy).

b) DNA content of sponges; ^{3}H Thymidine uptake into the DNA of the sponge and cylinder reparative tissue. These measurements are not specific for fibroblasts but are helpful when correlated with the histologic observations; special stains to aid in identification of fibroblasts are performed.

(3) Assessment of fibroblast function, collagen, ground substance, and blood vessel formation

a) Assessment of the quantity and morphologic appearance of the collagen on histologic examination of incisions and of the sponge and cylinder reparative tissue using special stains selective for collagen.

b) Various histologic stains to assess various components of ground substance qualitatively to assess blood vessel formation. (5)

c) Hydroxyproline content of the sponge and cylinder reparative tissue as a quantitative measure of reparative collagen.

d) Breaking strengths of the skin incisions in fresh state and also after fixation in formalin; the ratio of these two determinations reflects the degree of spontaneous cross-linking which has occurred in the wound collagen.

e) In selected cases, amino acid analysis of reparative collagen. (6)

II. Adverse Effects of Severe Injury on Wound Healing; Attempts to Improve Healing in the Severely Injured

Background

For some time, we have been conducting studies directed towards an understanding of the basic biochemical, physiologic and morphologic factors involved in wound healing and how these are affected by serious injury with the view of establishing the basis for improved prophylaxis and therapy of injured patients.

Normally, a complicated series of interrelated metabolic reactions exists in the young healthy adult in a manner that produces a relatively steady, albeit dynamic, state. These interrelationships are disturbed after injury and the steady metabolic state is modified. Changes involving protein, carbohydrate, fat, water, vitamin, endocrine and electrolyte metabolism in almost every organ on the body occur. The responses are dynamic and follow a generally predictable pattern (1-9).

Some of the usual features of the metabolic response to injury during the first several weeks may be briefly summarized as follows: increased heat production; weight loss; negative nitrogen, potassium, sulfur and phosphorus balances; increased gluconeogenesis, early hyperglycemia, and often modified carbohydrate utilization; altered fat metabolism, including increased concentrations of serum free fatty acids and a tendency to ketosis; retention of sodium chloride and water; and alteration in the metabolism of certain vitamins, e.g., ascorbic acid, riboflavin, thiamine, nicotinamide and vitamin A. The metabolism of certain trace elements, particularly iron, copper and zinc are changed in ways which may require increased intakes of these elements. These metabolic changes are usually accompanied in the early phase by involution of lymphoid tissue and the thymus, lymphopenia and eosinopenia, leukocytosis, adrenal cortical hypertrophy with associated depletion of adrenal cholesterol and ascorbic acid, and an increase in the secretion of certain sympathetic ganglia (norepinephrine), adrenal medullary, and adrenal cortical hormones and ADH. Whether thyroid and pituitary growth hormone secretions and functions change is unsettled. There is generally an early increase in serum glucagon and a fall in serum insulin; later serum glucagon falls and serum insulin rises. Liver function is altered, as evidenced, for example, by an increase in sulfobromophthalein retention and a decrease in the rate of conjugation of adrenal steroids.

A multiplicity of conditioning and modulating factors may modify these metabolic changes, including age, sex and nutritional status of the patients and the severity of injury. In general, the metabolic changes parallel the severity of the injury and are seen in their most accentuated form in previously healthy young men, in short, the wounded soldier.

Underlying these changes is a variety of factors including alterations in neuro-endocrine activity, fever, increased heat loss and heat production (primary and secondary), altered food intake, immobilization, effects of endogenous metabolites, effects of bacteria and their products when infection is present, and the external environment (temperature, humidity, air-flow). Attention should be drawn to the recent investigations of Wilmore, et al., suggesting that alterations in CNS and hypothalamic function resulting in increased catecholamine elaboration is fundamental to the hypermetabolism following injury (8, 9). The possible effects of environmental temperature on modifying the metabolic reactions to injury in animals (10) (11) (12) (14) (15) and patients (8) (13) has received considerable attention; we will return to this later.

Our understanding of these matters is still incomplete and we do not fully understand the mechanisms underlying these metabolic changes, nor do we fully understand their physiologic consequences. We will not discuss these matters in detail in this progress report since they are described elsewhere (1-15). We will focus in this report on the questions: How do these metabolic changes affect the clinical course and convalescence of the injured patients, particularly from the points of view of wound healing and wound infection? Should attempts be made to modify them?

(1) Effect of Injury on Wound Healing

We have pointed out elsewhere that since the injured man must heal his wounds for successful recovery, systematic studies of the healing of operative and traumatic wounds should provide objective evidence in one important area as to the significance of the early metabolic derangements. Although some have inferred that wound healing is "normal" in the early period after injury, the evidence with experimental animals is clear: wound healing is seriously impaired after injury. Thus, we showed a number of years ago that the healing of laparotomy wounds made in rats (16) and guinea pigs (17) one day after they were severely burned on their backs was significantly slower than in unburned controls.

A. Effects of Femoral Fracture on Wound Healing

We have extended this type of study to the healing of dorsal skin incisions and the formation of reparative tissue in subcutaneous implanted polyvinyl alcohol sponges to animals with unilateral and bilateral femoral fractures. The results of these experiments have recently been published in the Annals of Surgery (19) and the Journal of Trauma (14) (31). We turned to this experimental model for the following reasons: (1) There are considerable data in the literature regarding the metabolic responce of rats to femoral fracture; in fact, the early classic experimental studies of Cuthbertson carried out in the 1930's dealt with femoral fractures; (2) femoral fractures have some advantage over burns -- the water vapor loss through the burned area and presence of bacteria with possible infection of varying severity introduce variables which may complicate the study and its interpretation. Also studies in our laboratory over a period of years have shown that dorsal skin incisions and implanted polyvinyl alcohol sponges have certain advantages over laparotomy wounds.

These experiments were conducted with adult male rats. We found that the healing of skin incisions (e.g. breaking strength in both the fresh and formalin fixed states) and the formation of reparative tissue (e.g. hydroxyproline content) in s.c. implanted polyvinyl alcohol sponges are impaired significantly in rats with unilateral or bilateral femoral fractures (18) (19). This impairment in healing was greater in those rats with bilateral femoral fractures than in those with unilateral femoral fractures as judged by the formation of reparative collagen. The rats with fracture showed an expected significant increase in urinary nitrogen excretion. In some experiments the ratswere fed ad libitum while in others pair-feeding was carried out. The results were similar in both instances.

In other studies carried out a number of years ago, using N^{15} glycine, we found that the protein contents of the very active organs, such as the intestinal tract and liver, changed little or increased in the burned rats despite their markedly increased turnover rates. By contrast, the protein content of the carcasses of the burned rats decreased, and indeed, carcass protein accounted mathematically for most of the extra urinary nitrogen loss. This suggests that the integrity of certain vital organs is maintained at the expense of less active areas, such as the skeletal muscle (20). This view is supported by our observation (20) that liver regeneration following partial hepatectomy proceeds faster in the burned rat at a time when the healing of a laparotomy wound is impaired.

The difference in behavior of the liver and carcass of the burned rat is strikingly different from the behavior of these organs in the acutely starved but otherwise uninjured animal. There, liver protein drops sharply, at a rate far greater than that for the carcass protein in the starved but otherwise healthy rat. When rats are burned in addition to being starved, the loss of liver protein is slowed, as would be anticipated from 15_N glycine and liver regeneration studies (20).

These experiments suggest that both increased anabolic and catabolic processes go on after severe injury to the rat; all tissues do not participate equally in this response. These findings have just recently been confirmed by others (21). The available information on how patients react from the point of view of tissue protein synthesis and breakdown is very limited (22 - 28) due to the complexity of the interpretation of such studies where reliance must be placed almost entirely on blood, urine and fecal data, since tissue biopsy (other than of skeletal muscle, bone marrow, and skin) for research purposes is interdicted in most instances in patients. We have discussed these matters at length elsewhere, including the role of ascorbic acid in those animals (including man) which require exogenous vitamin C (17) (20) (29). We are aware of the work of Waterlow and his colleagues (22) (23) (32) suggesting that a primary change is decreased protein synthesis (e.g. of albumin) after injury, but the types of patients studied by them (patients undergoing modest or moderately severe elective surgery) and the timing (the first few days after injury) are quite different from the previously healthy young man undergoing severe traumatic injury. Recently Skillman and his associates (28) reported that daily postoperative infusion of a 3.5% solution of essential and non-essential amino acids led to a greater rate of albumin synthesis than the infusion of 5% glucose daily as tested on the 4th postoperative day in patients undergoing elective gastro-intestinal surgery.

B. Effect of Testosterone Propionate on Wound Healing in Rats with and without Femoral Fracture

In related experiments (published in detail recently (14) (31), we showed that the administration of testosterone propionate (s.c., or i.m.) to rats had no influence on wound healing of rats with or without femoral fracture. We conducted these experiments because we(12) and others, e.g., Abbott and Hirschfeld (13) had shown that the increased urinary nitrogen excretion which characteristically follows injury (and the intensity of which is proportional to the severity of the injury) is lessened by the administration of testosterone propionate to experimental animals and patients. However, there had been no evidence whether this was of any physiologic or clinical benefit. Our data indicate that it is not from the point of wound healing - that is, the impaired healing of rats with femoral fracture was not improved at all by administration of testosterone propionate beginning on the day of wounding and fracture and continued throughout the postoperative period.

C. Effect of Environmental Temperature on Wound Healing in Rats with and without Femoral Fracture; Effect of Testosterone Propionate at two Environmental Temperatures

The findings of these studies have very recently appeared in the J.Trauma (14) (31); a preliminary report had been presented elsewhere.

Caldwell and his associates (34) (35) (36) and Cuthbertson and his associates (37) (38) (39) have reported that the increased urinary nitrogen excretion of rats with burns (Caldwell) or femoral fracture (Cuthbertson) is sharply reduced when they are adapted to a higher than normal ambient temperature (30 °C vs. 22 °C) prior to injury and maintained at the higher temperature. Cuthbertson also reported a limited experiment which suggested that this was also so when rats maintained at 22 °C prior to injury were transferred to 30 °C immediately after injury.

Our experiments have shown that when rats in a 22 °C room with or without femoral fracture were transferred just after injury or sham-injury to a 30 °C room, there was a marked drop in food intake immediately. Accordingly, we have conducted both pair-feeding and ad libitum feeding experiments. Among pair-fed rats without fracture, wound healing was little different at 22° and 30°C. Femoral fracture impaired significantly healing of skin incisions and collagen formation in s.c. polyvinyl alcohol sponges when rats were kept at 22° postoperatively, but this difference was much less for rats at 30 °C. Wounded rats with fractures ate less after injury when offered food ad libitum particularly those kept at 30 °C. Despite this, weight losses were similar for rats at 22° and 30°. When the rats of all groups at 22° and 30° (with and without fracture) were pair-fed against rats with fracture at 30 °C., weight loss was less for rats at 30 °C and their urinary nitrogen excretion relative to nitrogen intake was less than that by corresponding rats at 22°. At 22°, fractured rats excreted significantly more nitrogen relative to food intake than pair-fed sham controls, but at 30°C., this ratio was similar for rats with and without fracture.

Testosterone propionate, s.c., decreased urinary nitrogen excretion by rats with femoral fracture maintained at 22° or 30° after injury, but had no salutory effect on wound healing in those rats or their pair-fed sham-fractured controls. In fact, testosterone slowed healing of the rats with fracture, particularly as judged by the collagen contents of the sponge granulomas.

D. Studies with Vitamin A, Vitamin A Antagonists (Citral), DOCA, and Metyrapone; Cortisol Receptor Protein

Background

We pointed out in our previous reports that a disturbance in the metabolism of water-soluble vitamins, especially ascorbic acid, after serious injury is well documented, but that little information regarding the fat-soluble vitamins after injury is available (40,41,42,43,44-48,49,50,50a).

A number of observations in our laboratories during the last few years have suggested that there may be an increased requirement for vitamin A after injury under conditions where pre-existing vitamin A deficiency does not exist. We began these experiments on the hypothesis that serious trauma increases the requirement for vitamin A. Kagan (51) had found that rats with subcutaneous abscesses as the result of repeated injections of turpentine and sweet almond oil showed sustained decreases in serum vitamin A concentration and in liver vitamin A concentration and content. Gastrointestinal absorption of vitamin A was not altered, but urinary excretion increased, and the kidney concentration of vitamin A was higher than normal. Clark and Colburn (52) had found that large doses of cortisone result in the rapid depletion of vitamin A from the liver and kidneys of rats. Hunt and his associates (53) (54) have shown that vitamin A restored the breaking strength of incisions and the closure of open skin wounds in cortisone treated rats towards normal, but the vitamin A had no positive effect on the healing of rats not receiving cortisone. Our experiments suggest, however, that vitamin A has an accelerating effect on wound healing in rats eating a nutritionally complete commercial rat chow and not given cortisone. Herrmann and Woodward (55) also found that vitamin A given to "non-deficient" rats resulted in increased fibroplasia as judged histologically and by hydroxyproline content of sponge granulomas.

Recently, there have been reports (including our own (56,57, 58) of an apparent increased need for vitamin A after injury, with particular reference to the occurrence of stress ulcers (59), burns(60,50a)and fracture (56,57,58).

1. Effect of Marginal Vitamin A Intake on Response to Minor Wounding

Rats on a marginal intake of vitamin A, sufficient to maintain near normal growth, lost weight promptly after relatively minor wounding (dorsal skin incision and polyvinyl alcohol sponge, s.c., implants) despite little difference in food intake compared with their wounded normal controls and about half of the rats on marginal vitamin A intake died. The mildly A-deficient rats formed less reparative collagen, and that collagen which was formed was less cross-linked than normal (as judged by the ratio of the breaking strengths of wounds treated in the fresh state and after formalin fixation) providing for a much weaker wound.

No differences were found between the two groups of rats in terms of blood urea nitrogen (BUN) and the serum Na, K, Ca, Cl., uric acid or creatinine concentrations, all of which were within normal limits. The serum glucose, however, was 65 mg. percent in the deficient rats versus 120 mg percent in the controls. It is possible that impaired glucose mobilization may be associated with the reduced ability of the vitamin A deficient rat to survive surgical stress. This may involve an interference with either corticoid or growth hormone responsiveness in the A-deficient state.

In another experiment, rats were rendered vitamin A-deficient as described above. After wounding, the deficient animals lost weight at a rate similar to that seen in the previous experiments. At the end of the first week postoperatively the animals were treated each day for the next 14 days with two times the daily vitamin A intake of the control rats used in this study. There was an immediate response to the vitamin A. The sluggish, moribund rats became more active, gained 8 grams in the first day and continued to gain weight at a very high rate for the following week. When they were killed (21 days postoperatively) their wounds were as strong as those of control rats when tested in both the fresh and formalin-fixed states. We carried out this study prior to the present progress report;it is included for background information. (56)

2. Effect of Vitamin A Administration to Wounded, "Non-deficient" Rats

Supplemental vitamin A (oral and topical to margins of skin incisions and instilled in the sponges at the time of implantation) given to healthy normal rats ingesting a commercial rat chow, but restricted in food intake by pair-feeding, subjected to dorsal skin incisions and polyvinyl alcohol sponge s.c. implants, led to significant increases in wound strength ($p < 0.001$).

3. Effect of Vitamin A Supplementation on the Healing of Wounds of Rats with Femoral Fracture (46) (58)

a. Incisions, b. Implanted polyvinyl alcohol sponges

Groups of healthy wounded rats with and without unilateral or bilateral femoral fractures fed a commercial chow ration were studied with and without the addition of supplemental vitamin A. In some experiments the animals were pair-fed and in other experiments were allowed food ad libitum. The healing of dorsal skin incisions and the formation of reparative tissue in s.c. implanted polyvinyl alcohol sponges were significantly increased when supplemental vitamin A was given, although the vitamin A did not completely obviate the adverse effects of fracture (58). The ratio of the breaking strengths of the skin incisions after formalin fixation to the breaking strengths in the fresh state was lower in the vitamin A supplemented rats, supporting our earlier experiments that vitamin A increased the rate of collagen cross-linking.

For histologic evaluation of the skin incisions and sponge granuloma of the rats killed at 11 days, a quantitation for each category described(collagen, ground substance and number of fibroblasts) was based on arbitrary scales of 1 to 3 and the ratios of ground substance to collagen calculated. No statistically significant differences in collagen and ground substance were found by the examination of skin wounds. Ground substance of sponge granulomas of vitamin A fractured rats compared with peanut oil group was significantly increased ($p < 0.02$) as was the ratio of ground substance to collagen ($p < 0.001$). No differences were found in the number of fibroblasts in the wounds or sponge granulomas.

c. Open Skin Wounds

In contrast to our findings regarding skin incisions and the formation of sponge granulomas, unilateral or bilateral femoral fracture did not slow the rates of closure of open dorsal skin wounds, nor did supplemental vitamin A have any effect on closure of open wounds in rats with or without femoral fracture.

These differences in behavior between the healing of sutured skin incisions and the closure of open skin wounds are not too surprising. The healing of open skin wounds depends on factors different in several important ways from

those involved in the gain of strength of healing skin incisions. Thus, while little strength is attained by healing skin incisions in scorbutic guinea pigs (in large measure due to a failure of collagen synthesis) the closure of open skin wounds in such guinea pigs proceeds at an essentially normal rate as shown by Abercrombie and associates (61) and Grillo and Gross (62).

It should be noted, though, that Hunt and his associates found that the closure of open wounds in rats treated with cortisone is slowed and this is partially obviated when supplemental vitamin A is given locally or parenterally. Thus, the effects of exogenous cortisone on the closure of open skin wounds are different from what we have observed in rats with femoral fracture. Whether the difference is largely quantitative (i.e., a reflection of the dose of cortisone used versus the stimulus of femoral fracture) or qualitative (i.e., a reflection of qualitative differences in metabolic and physiologic changes due to exogenous cortisone versus the effects of femoral fracture) remains to be established.

4. Experiments with Vitamin A Supplementation of Defined Diets on Wound Healing

We have carried out several experiments 1) with rats initially fed a commercial rat chow and then adapted to a vitamin A deficient (defined) diet supplemented with added vitamin A,and 2) with rats fed from weaning the defined diet supplemented with vitamin A. After being subjected to a dorsal skin wound, polyvinyl alcohol sponge implants and bilateral comminuted femoral fractures, oral and topical (to wounds) vitamin A in varying amounts was given to the rats. The intake of more vitamin A did not prevent postoperative weight loss after fracture and wounding, particularly among rats maintained on the defined diet from weaning. No differences in healing were found among rats adapted to the defined diet with incisions. We raised the possibility in our research application that perhaps the failure of vitamin A to ameliorate the healing of wounds in rats with fracture ingesting the defined diet in contrast to the positive effect of vitamin A given to similarly injured and wounded rats ingesting a chow diet might be the presence of some nutrient(s) in the chow diet absent perhaps in the defined diet. One such group of nutrients we considered were the trace elements, since, for example, (1) in the absence of adequate zinc, vitamin A is not mobilized from the liver and (2) when body zinc is low, healing is impaired. Accordingly, an experiment was conducted recently in which trace elements known to be required by the rat were added in amounts considered adequate even in the presence of injury. Irrespective of the oral supplements or topical vitamin A treatments, supplemental vitamin A minimized the characteristic early rise in adrenal weight associated with injury. Liver vitamin A concentration and contents were similar for a given supplemental dose of vitamin A in rats only wounded and rats wounded and subjected to femoral fracture. Serum vitamin A concentrations were lower in wounded rats with fracture than in rats only wounded at each level of vitamin A supplementation. Within each of these groups, the level of vitamin A supplementation had little effect on serum vitamin A despite differences in liver vitamin A contents. In this respect, the injured rats behaved relatively similarly to the uninjured animals. No difference in the strength of 10-14 day old dorsal skin incisions was noted among various groups of rats each maintained from the time of weaning (3 weeks of age) on a vitamin A free diet supplemented with 50-60 μg vitamin A three times a week for 8 weeks, then subjected to dorsal skin incisions and s.c. polyvinyl alcohol sponge implants; postoperatively some groups were maintained on the level of 50-60 μg vitamin A while other groups were given 1000 μg vitamin A. We believe that the failure of vitamin A to affect the gain of wound strength in the rats with femoral fracture ingesting the "defined" diet while it does in rats with femoral fracture ingesting a "nutritionally complete" commercial rat chow diet reflects a

nutrient deficiency in the "defined" diet which we have not yet identified. The histologic examinations of the incisions and sponge reparative tissue and the hydroxyproline measurements of the sponge reparative tissue are in progress and will be reported in a subsequent progress report.

5. Effect of Vitamin A and Citral on Peritoneal Adhesion Formation

Background

The development of adhesions following intraabdominal surgery constitutes an important surgical problem. Adhesions are a major cause of mechanical intestinal obstruction, often requiring operative therapy. The mechanism of adhesion formation remains unknown. Several factors which influence their development are: trauma, foreign bodies, and bacterial infection. Ellis (63) has proposed that it is not serosal integrity but tissue ischemia which is the important factor in the etiology of postoperative adhesions.

We have mentioned earlier in this report that Ehrlich and Hunt have shown that vitamin A antagonizes the inhibitory effect of cortisone on wound healing(53) (54). We have also pointed out that we have shown that supplemental vitamin A accelerates healing of skin incisions in normal rats and rats with femoral fracture ingesting rat chow (58). Another observation made in our laboratory is that rats receiving high doses of vitamin A orally for periods of 4 - 5 weeks, exhibited thickening of the splenic and liver capsules. Based on these observations, we decided to study the effect of vitamin A and a vitamin A inhibitor on the formation of intraabdominal adhesions.

Experiments were carried out on Swiss, white female mice, weighing 20-25 grams. Peritoneal ligation as described by Ellis (64) was the method used to produce intraabdominal adhesions. Open ether anesthesia was used. Sham-operation consisted of opening and closing the abdomen through a midline incision. Animals were given ground laboratory chow and water ad libitum. In the experimental groups of mice, the diet was supplemented with either all-trans-Vitamin A acetate or Citral or both. Citral is a vitamin A inhibitor; its chemical formula is:

$$(CH_3)_2CCHCH_2CH_2C(CH_3):CHCHO.....M.W.:152.24$$

The technique of peritoneal ligation proved to be a simple and reliable method for the production of intraabdominal adhesions. From our experimental results it was evident that the ingestion of a diet with a high (300 mg/Kg diet) or intermediate (150 mg/Kg diet) supplement of vitamin A for ten days following peritoneal ligation enhances adhesion formation whereas a lower (75 mg/Kg diet) supplement of vitamin A had no effect. On the other hand, ingestion of a diet with a high supplement (10,000 γ/Kg diet) of Citral for ten days postoperatively prevented adhesion formation in a significant number of mice. An intermediate dietary supplement of Citral (1,000 γ/Kg diet) had a lesser but significant effect, whereas a low (100 γ/kg diet) supplement had no effect. Simultaneous administration of dietary supplements of vitamin A and Citral produced variable results: in the high supplement group , the vitamin A effect was greater than that of Citral, resulting in the development of 3+ adhesions in over 50% of the animals. However, Citral prevented the full expression of the vitamin A effect as shown by the absence of 4+ adhesions (53% of the mice receiving this level of supplement of vitamin A alone developed 4+ adhesions). In the intermediate supplement group, the Citral effect was more pronounced, 40% of the animals had 1+ adhesions and none had 3+ adhesions (40% of the mice receiving vitamin A only had 3+ adhesions.)

In the low supplement group, there was no significant difference from the controls (80).

6. Effect of DOCA administration on vitamin A and Wound Healing

The administration of modest amounts of DOCA to rats mildly deficient in vitamin A (as result of a low intake of vitamin A) results in severe weight loss, marked decrease in strength of healing wounds and decreased formation of implanted sponge granuloma collagen. All these effects are much greater than are seen in vitamin A supplemented rats given DOCA. We have mentioned that Ehrlich and Hunt have shown that extra-vitamin A antagonizes the adverse effects of cortisone on the healing of incisions and open wounds in rats (53, 54).

7. Effects of Metyrapone

Background

The marked metabolic derangements associated with injury include several reactions which are similar to those which result from excess glucocorticoid hormone secretion. Two such reactions are muscle-wasting and decreased immunologic competence. We (15) (20) and others (2)(4)(9)(12)(13)have discussed in detail elsewhere current views regarding the role of the CNS-Hypothalamus-Pituitary-Adrenal Axis in the metabolic reaction to injury. Metyrapone inhibits glucocorticoid synthesis and leads to the accumulation of DOC. In experiments using Metyrapone we have found the following:

Rats

1) In rats subjected to dorsal skin incisions and implantation s.c. of polyvinyl alcohol sponges, we have always observed postoperative weight loss which persists for several days. We have observed that administration of metyrapone (200 mg/kg diet) decreases this weight loss.

2) The operation causes a 35% reduction in spleen size; metyrapone treated animals show only a 10% decline ($p < 0.001$).

3) Operated animals show a 25% decrease in thymic size; metyrapone animals showed no loss in thymic weight ($p < 0.001$).

4) The breaking strength of the incisions of the metyrapone treated animals was significantly higher than that of the control rats ($p < 0.001$), and the metyrapone treated rats formed reparative collagen (as assessed by hydroxyproline) at a faster rate in s.c. implanted polyvinyl alcohol sponges.

Mice

1) In normal mice, metyrapone increased thymic weight slightly, and thymic protein and RNA substantially (65).

2) Physical stress (the wearing of a body cast, such as used in skin graft experiments) decreased thymic weight, protein, DNA, and RNA; metyrapone antagonized these effects significantly.

3) Physical stress decreased the immunocompetence of mice as judged by their ability to reject allografts and a viral tumor (Moloney Sarcoma); metyrapone increased the immunocompetence of both stressed and non-stressed animals as judged by their resistance to inoculation of the Moloney Sarcoma virus (lower tumor incidence, prolonged latency period, and faster tumor regression time) and C_3H breast adeno carcinoma(tumor incidence, tumor score, survival) (66).

4) Metyrapone antagonized stress ulcer formation following restraint and food deprivation (67).

8. Effect of Vitamin A on Cortisol Receptor Protein

In looking for an explanation for the observations of Hunt and his coworkers, that vitamin A can antagonize some of the defects in wound healing due to cortisone administration and our own observation that vitamin A can overcome some of the adverse effects of trauma in wound healing, we examined the influence of vitamin A on the cortisol "receptor" protein.

The cortisol receptor protein is not to be confused with the cortisol binding globulin (transcortin) present in plasma. The receptor protein has a much higher affinity for the steroid and is thereby able to accept transcortin-bound steroid. Liver and thymus are the two richest sources of corticol receptor protein.

Significance of the cortisol receptor protein

The cortisol receptor protein permits liver and thymus to remove cortisol from the circulation and permits the liver to respond to cortisol (e.g. induction of enzymes for gluconeogenesis) and the thymus to respond to cortisol (selective destruction of non-sensitized lymphocytes; inhibition of protein synthesis by surviving lymphocytes). Prior to our work it was known that liver and thymus levels of the cortisol receptor protein were elevated by cortisol administration and lowered by adrenalectomy.

Our preliminary experiments show the following:

(1) The stress of partial body casting (the type of stress that is immunosuppressive as assessed, e.g., by allograft rejection and Moloney Sarcoma Virus tumor rejection) more than doubles the liver content of cortisol receptor protein, and increases the cortisol receptor protein in the thymus to an even greater degree.

(2) Supplemental vitamin A (given to normal rats) increases the levels of the receptor protein about 20%, but vitamin A blocks the much larger (100-200%) increase in liver and thymus cortisol receptor protein induced by partial body casting.

Current Studies with Vitamin A, Metyrapone and Deoxycorticosterone (DOC)

We are continuing our studies with vitamin A in two major areas:

1. wound healing in seriously injured rats with emphasis on the mechanism(s) of action of vitamin A;

2. infection, local and systemic, with the view that supplemental vitamin A will increase the resistance (local and systemic) of seriously injured animals. This aspect of our investigations is described in Section IV of this report.

Studies with Metyrapone and deoxycorticosterone (DOC) will be carried out also in these two areas. These studies are designed to cast light on the mechanism of vitamin A's actions and are not conducted with the view that these agents (Metyrapone, DOC) will be used as therapeutic agents clinically in the care of injured patients.

Some of the Specific Experiments to be Conducted are Directed Toward Answering the Following Questions

(a) Does vitamin A require the presence of macrophages to exert its ameliorating effect on the impaired healing of seriously injured animals? Anti-macrophage serum will be used to help answer this question.

(b) A question related to the above is the following: Will vitamin A exert its antagonistic effect on exogenous cortisone given to rats whose only injury is a skin incision or open skin wound and who are treated with anti-macrophage serum or does vitamin A require the presence of macrophages for this antagonistic effect to be evident?

The major initial examination in both experiments (a) and (b) will include measurement of the breaking and tensile strengths of the skin incisions, the weight, hydroxyproline, DNA, and protein contents of the sponge (or cylinder) reparative tissue, and the histologic examination of the skin incisions and the sponge (or cylinder) reparative tissue. Depending on the findings, additional specialized measurements related principally to collagen synthesis and cross-linking will be done.

(c) Does vitamin A affect collagenase activity?

Collagenase activity in the reparative tissue (sponge or cylinder) will be measured in some groups of rats of the preceding experiments to test our hypothesis that vitamin A may affect collagenase activity; our rationale for this hypothesis was given in our research application. This hypothesis will also be tested in experiments in which some rats receiving exogenous glucocorticoids are given supplemental vitamin A while others are not. The breaking strength of the incisions will be measured also and the weight, hydroxyproline, DNA, and protein contents of the sponge (or cylinder) reparative tissue will be determined. Histologic examination of the healing incisions and reparative sponge (or cylinder) tissue till be carried out.

(d) Vitamin A Metabolism After Injury

1. We plan to continue our studies to:

   a. determine quantitatively the influence of injury on the metabolism of vitamin A,

   b. determine the level of increased requirement of vitamin A following wounding and other injuries.

The levels of vitamin A will vary for different groups of rats, matched by starting weight, and pair-fed or fed ad libitum postoperatively. The injuries will be unilateral or bilateral femoral fractures in some experiments; the test wounds will be skin incisions and s.c. implanted polyvinyl alcohol sponges.

(e) Vitamin A, Cortisol Receptor Protein

1. determine the effect of unilateral and bilateral comminuted femoral fractures and severe burns (30-35% body surface, 3°) on the concentration and content of cortisol receptor protein in the liver and thymus of rats;

2. determine the effect of supplemental vitamin A given to similarly injured rats on the liver and thymus cortisol receptor protein.

Roles of Vitamin A, Metyrapone: Deoxycorticosterone and Adjuvants in Resistance to Infection

Our previous and proposed studies of the relationship of vitamin A, Metyrapone, deoxycorticosterone and certain adjuvants to infection, specifically in regard to the altered resistance to infection of severaly injured animals and patients are described in Section IV of this report.

E. Arginine Supplementation as a Stimulus for Wound Healing: Growth Hormone

Background and Rationale

Several years ago, we undertook a study aimed at determining the relative importance of dietary proline, glycine and arginine for wound healing. These experiments were based on the fact that these amino acids can be synthesized by the rat but under conditions of rapid body growth, there can be a growth response to dietary supplementation with these amino acids. Further, glycine is present in very high concentration in collagen and scleroproteins, and proline and its derivative hydroxyproline are present in high concentrations in collagen. Arginine is also present in fairly high concentration in collagen. In addition, arginine is a precursor of proline and, therefore, also of hydroxyproline.

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We were interested in studying the relative significance for wound healing of the following pathways:

a) glutamate-------> pyrroline carboxylic acid--------> proline

and

b) arginine------> ornithine------> pyrroline carboxylic acid---> proline

In an initial experiment designed to determine if glycine or proline stimulated wound healing, young adult Sprague-Dawley rats were wounded in a standard fashion (dorsal skin incisions and subcutaneous implants of polyvinyl alcohol sponges) and placed on a chemically defined diet lacking proline, glutamic acid, glycine and arginine. These animals were placed in one of four groups: (1) no supplement, (2) glycine supplemented, (3) proline supplemented, and (4) glycine plus proline supplemented.

During the first postoperative week all the wounded animals lost 15-20 g each. It should be emphasized that unwounded rats of this age gain weight when ingesting these diets. All diets were then altered to include an arginine supplement, whereupon the wounded animals began to gain weight at a near normal pattern. Under these conditions, then, arginine was a major limiting amino acid for the wounded, but not the unwounded rats. Adding glycine to the arginine supplemented diet in comparable circumstances led to an additional modest weight gain so that the rats now gained weight normally. When proline was added rather than, or in addition to, glycine, no effect in weight gain attributable to proline was seen.

In terms of wound strength, there was a substantial delay in healing in all groups during the first week postoperatively. After the arginine supplement was added, there was an abrupt increase in the rate of gain of wound strength so that near normal levels were reached in 2-3 weeks. Additional proline, added to the arginine, had no apparent additional effect on the already improved healing, but when glycine rather than proline was added to the arginine supplemented diets, healing was accelerated still further, just as the gain in weight was. The results show that under the conditions of these experiments arginine is indispensable for both growth and wound repair of wounded animals; the arginine requirement is clearly increased by the wounding, since unwounded young adult rats grow satisfactorily on the unsupplemented basal diet.

We have recently conducted several experiments to study the influence of arginine supplementation on wound healing in young adult rats fed a commercial laboratory chow (Teklad)*. In a typical experiment two groups (10 male rats each, weighing 190 g) were maintained on Teklad. Three days preoperatively the ration of one group was altered as follows: to the chow, arginine-HCl was added, 5 g/kg diet, and their drinking water was made to contain 0.5% arginine-HCl. The other group of rats (controls) was continued on the unmodified chow and tap water. After wounding (dorsal skin incision and the subcutaneous implantation of polyvinyl alcohol sponges) the controls were continued on their preoperative food and water rations, while the experimental group was fed the arginine supplements for an additional 4 days, and then fed the control rations. The rats were killed 10 days postoperatively. The breaking strengths of the dorsal skin incisions of the arginine supplemented group were significantly stronger (75%, $p < 0.001$) than those of the control unsupplemented group. This is a remarkable effect.

* This chow contains 1.8% arginine and normal rats grow and reproduce in excellent fashion when ingesting it.

We have confirmed this arginine effect in several subsequent wound healing studies (68,69,70).

We believe that the arginine effect on healing is due to the following:

a. it is required for repair and regeneration in amounts that cannot be synthesized by the rat, i.e., there is an increased arginine requirement resulting from wounding or injury;

b. the added arginine may act by becoming incorporated into protein(s) as either arginine or proline or the arginine may be acting by giving rise to diamines (putrescine, agmatine) or polyamines (spermine, spermidine) which may stimulate fibroblast division or which may influence inflammation and neovascularization;

c. the added arginine may act as a releasing agent for hormones, such as growth hormone, insulin and glucagon, or it may act to inhibit the release of ACTH.

In an initial experiment using hypophysectomized rats receiving ACTH, testosterone propionate, and thyroxine, but not growth hormone, we (69) have found that dietary arginine supplementation did not affect wound healing (breaking strength of dorsal skin incisions and hydroxyproline content of the reparative tissue found in s.c. implanted polyvinyl alcohol sponges). This experiment supports the view that the arginine effect on healing may be mediated by growth hormone. We are currently repeating and entending this type of study using hypophysectomized rats which receive hormonal supplements other than growth hormone, some of which receive arginine supplementation and some of which do not. The effects of the arginine supplementation on healing will be assessed using the methods already described in this report. We anticipate confirming our initial experiment and will then extend the experiments to include hypophysectomized rats receiving ACTH, testosterone propionate, thyroxine and growth hormone (some groups in maintenance amounts, other groups in supermaintenance amounts) some of which receive arginine supplementation and some of which do not. We have reviewed in our research grant application the studies of others dealing with the effects of growth hormone on wound healing and protein metabolism after surgery.

Our current experiments are aimed at determining the extent of the arginine effects on healing in severely injured animals and the mechanism of the effects. In these experiments, in addition to observations of wound healing, we plan to determine: (a) plasma growth hormone levels in wounded and unwounded rats with and without bilateral fractures and with and without arginine supplemente; (b) plasma corticosterone in the same animals; and (c) liver and thymic cortisol binding protein levels in these animals.

Our current studies also deal with the effect of arginine on wound healing in seriously injured animals; growth hormone supplementation with and without arginine supplementation will also be assessed. Rats with bilateral femoral fractures and control rats without fractures will have skin incisions, polyvinyl alcohol sponge s.c. implants or stainless steel mesh cylinder implants. Groups of rats will be treated with arginine and growth hormone, singly and together. Appropriate untreated control rats will be studied concurrently. Dietary intake will be ad libitum in some experiments, pair feeding, in others, and when indicated, regulated by tube feeding. Measurements of body weight and the healing of the skin incisions and the formation of the sponge or cylinder reparative tissue will be assessed by the techniques already described. Parallel studies will be carried out with rats with burns (30-35%, third degree) rather than femoral fracture, as described elsewhere in this application.

Extension of our observations on the effect of growth hormone will be made, with special attention directed toward the influence of this compound on collagen synthesis. The hormone will be added to culture media of fibroblasts at various concentrations. Incubation with proline-^{14}C will then be done with both exponentially growing and stationary cultures, in order to establish whether the effects of growth hormone on collagen synthesis are different in rapidly growing and in non-growing cell populations.

F. Action of Tumor Angiogenesis Factor(s) (TAF) in Healing Wounds. Can such Treatment Promote the Healing of Wounds in Severely Injured Animals

Background

Folkman has described the preparation of a protein from various solid tumors and from tumor cells grown in culture which has the property of stimulating angiogenesis (TAF) (71-75). The protein has been extracted from both the nuclei and cytoplasm of a variety of tumors and the current view is that TAF is a non-histone protein produced in the nucleus, and is stored in and secreted by the cytoplasm. Its molecular weight is in the neighborhood of 100,000. Not all workers accept the view that TAF is uniquely a tumor protein. Proteins of similar function have been associated with vascularization in fetal development and in vascularizing wounds. In the healing wound several groups (Ross (76), Hunt (77), and Peacock (78)) have found that macrophages are intimately involved in wound repair by their support of fibroplasia. Hunt's group has shown that the macrophage stimulates wound angiogenesis, although they have not shown that macrophage extracts can do so.

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Folkman and we have shown that TAF is not tumorigenic and we have found that its use does not confer immunity to tumors such as MuSV. Since angiogenesis is a limiting factor in the early wound healing period, we have been carrying out experiments to determine how TAF affects fibroplasia, including fibroblastic proliferation and collagen synthesis.

We have used murine solid tumors of two types as sources of TAF: (a) C3HBA (malignant mammary tumor) and (b) MuSV-M (Murine Sarcoma virus tumor). For our first experiments, the solid tumors were harvested and extracted for our initial experiments by the original procedure of Folkman (71,72)

(1) Two experiments were conducted using such TAF prepared from MuSV-M tumors:

1. Rats were subjected to dorsal skin incisions for the subcutaneous implantation of polyvinyl alcohol sponges. The sponges contained either muscle extract, prepared by the methods used to prepare TAF (control), gelatin (additional control), or TAF in gelatin. After five days, the sponges were removed. TAF sponges had more than twice as much hydroxyproline as the other two groups. In another similar experiment using another TAF preparation, the sponges were removed at day 7. Again, the TAF sponges had more than twice as much hydroxyproline as controls. The gelatin was absorbed by this time.

2. Dorsal skin incisions were made in rats and the incision bathed with a solution of TAF in Hanks' solution just prior to suturing. A control group of rats were operated upon but a similar solution of muscle extract used rather than TAF. All rats were killed 2 weeks later. No effects on breaking strength of the skin incisions were found. In a subsequent experiment, the solutions were instilled in the wound area for 3 days and the rats killed on day 8. The breaking strength of the wounds of the TAF treated rats was now twice as strong as that of the control rats.

(continued next page)

(2) Three experiments were conducted using TAF prepared from C3HBA tumor, a tumor requiring cell transplantation and presumed not to depend on the presence of an oncogenic virus.

1. The extracts were evaluated using the Selye air pouch technique. Increased capillarity of the pouch was seen in rats injected with the extract confirming Folkman who also used the air pouch technique (5). Muscle and liver extracts did not enhance capillarity. We found increased blood vessel development at days 2, 3 and 5.

2. This type of TAF preparation was used by Drs. H. Miller and G. Sosler in our department in dogs subjected to coronary artery constriction to see if TAF treatment increased collateral vessel development. Application of TAF in undissolved gelatin and agar increased vascularization of the myocardium more than application of gelatin and agar alone did. The dogs were followed and observed 3 and 6 weeks after the application of an Ameriod constrictor.

3. Rats were subjected to 7 cm dorsal skin incisions and to implantation of polyvinyl alcohol sponges into subcutaneous tissue under pentobarbital and ether anesthesia. The wounds were treated with either TAF or an extract of non-tumorous muscles (control). Animals were killed on postoperative days 6 or 10. The mean breaking strength for 10 day old control wounds was 190 gm; wounds from TAF treated animals were stronger, 272 gm, $p < 0.002$. Granulomas from control rats contained 0.27 mg of hydroxyproline/100 mg sponge at day 6 and 1.25mg/100 mg sponge at day 10. The corresponding values for the TAF treated groups were 0.6 mg at day 6 ($p<0.001$) and 1.8 mg at day 10 ($p<0.001$). We conclude that TAF supports increased fibroplasia in the rat presumably through TAF-stimulated neovascularization, but the mechansim remains to be found (79).

We plan to extend these promising studies. Our studies will be directed towards answering the following questions:

(a) Can the action of TAF on wound healing be modified (improved) by use of techniques to alter the rate at which TAF is delivered to a wound site?

(b) Can TAF be used to promote healing of wounds other than skin incisions, e.g. skin grafts?

(c) Can TAF be used to help correct the impaired healing of wounds associated with severe injury?

(d) How does TAF function to promote angiogenesis, fibroplasia and increased collagen synthesis? Can TAF action be accentuated by combining it with the use of chemicals such as vitamin A which also have local effects on promoting healing?

II (2) Additional Studies Dealing with Attempts to Increase Fibroblastic Proliferation, Collagen Synthesis and/or Collagen Cross-linking and Thereby Accelerate Healing After Injury .

The critical role fibroblasts play in wound healing has long been recognized. Without adequate numbers of fibroblasts, without properly functioning fibroblasts, wound healing cannot proceed. These cells, among their special functions, are the sole manufacturers of collagen, and the synthesizers of some of the essential components of the ground substance. The adverse effects of "too much" cortisone on wound healing, for example, are due in large measure to a substantial decrease in the numbers of fibroblasts due in part to inhibition of their multiplication and some changes in their ultrastructure. In ascorbic acid deficiency, there is no decrease in the number of wound fibroblasts, nor in the rate in which they appear, but there is a significant change in the ribosomal arrangement along with the endoplasmic reticulum, a disarrangement which is promptly restored to the normal orderly "polysomal" arrangement when ascorbic acid is given - Ross and Benditt (1), Ross (1a).

Fibroblasts have generally been considered to begin making collagen only after the "growth" phase but recent results from our laboratory indicate that fibroblasts in the logarithmic growth phase can synthesize and extrude collagen (Manner) (9).

The rates at which the entrance and multiplication of fibroblasts enter the wound under normal circumstances are well known. These rates are slow enough to have encouraged investigators to attempt to accelerate them, with the expectation that wound healing would be speeded up thereby. Prudden has reported limited success in this regard with cartilage powder and n-acetyl-glucosamine polymers applied locally to the wound (2).

a) Additional Methods to Increase Fibroblast Proliferation and Collagen Synthesis in Wounds. A1). Implantation of Cultured Fibroblasts into Animals

Our objectives in this phase of our studies were to determine whether cultured diploid fibroblasts survive when added to polyvinyl alcohol sponge implants in rats, remain viable upon reincubation, accelerate the synthesis of ground substance and collagen, and increase the rate of healing and the strength of wounds. The long term fate of implanted cultured diploid fibroblasts was also studied, with particular attention to possible tumor formation.

Background

The survival of and the reactions to reimplanted cultured cells in animals or patients have been studied by a number of investigators. Most of these studies were, however, concerned with the investigation of rejection phenomena and/or of specific tumor antigens. Southam et al. (1) showed that human and other neoplastic cell lines grew better after subcutaneous injection into cancer patients than in healthy volunteers; chick embryo fibroblasts did not survive (3). Established epithelial cell lines from normal tissues also survived well after subcutaneous injection into cancer patients, but such established lines at the time of implantation were no longer diploid (Moore et al., 1956 (4)). Cells of the human epidermal line NCTC 3075 survived for several months in patients with advanced mycosis fungoides but for shorter time periods in patients in less advanced stages of the disease, indicating an impairment of the immunological defense mechanism (Van Scott et al. (1956) (5). In these studies, the implanted epithelial cells did not keratinize or otherwise carry out specialized functions of differentiated cells. Billingham

and Silvers (1970) (6) found that pigmented cells of guinea pig cells survived well when transferred into non-pigmented areas within the same animal or a sibling within a highly inbred strain. Human skin epithelial cells grown in culture can be successfully autotrnasplanted to burned patients ( ); the long term fate of these transplants has not been determined, i.e., whether neoplasms may develop.

a. Tissue Culture of Fibroblasts

Animals of highly inbred strains (Fischer rats) were used in order to obviate genetic variability.

Fibroblast cultures were derived from the skins of newborn animals by mincing and trypsinization (0.1 - 0.25% trypsin, at times in the presence of 0.001 - 0.002 M Na_2 EDTA). The cultures were grown in Petri dishes in Eagle's MEM with 5 - 10% calf serum and subcultured until enough material for the implantation experiments was available. For the experiments, the cells were dispersed by trypsinization, washed repeatedly with balanced salt solution to remove calf serum proteins, and finally suspended in balanced salt solution for reinjection into experimental animals. Aliquots of the suspension were also transferred into Petri dishes with the standard growth medium in order to determine the viability of the suspended cells at the time of reinjection.

b. Determination of Survival and Function of the Cultured Fibroblasts When Injected into Animals; Effect on Wound Healing (Manner et al.(9), Seifter et al. (8).

In our investigations the effect of the implantation of Fischer rat fibroblasts grown in culture on a) the rate of collagen synthesis in subcutaneously implanted "Ivalon"polyvinyl alcohol sponges and b) the healing of skin incisions was studied in rats.

a) Rat embryo skin fibroblasts were derived from the dorsal skin of 15-17 day rat embryos. Fibroblasts from reparative tissue were obtained from polyvinyl alcohol sponges subcutaneously implanted into rats and removed after 4-5 days. Dulbecco-Vogt medium containing 0.025 M of HEPES buffer (pH 7.35) and supplemented with 10% fetal calf serum, penicillin, streptomycin, amphotericin B, and aureomycin, at 36°-37°C in 10% CO_2/air, were the conditions of cell culture.

The cells were harvested by trypsinization, washed twice by centrifugation in cold serum-free culture medium, and were then resuspended in serum-free medium ($1-2 \times 10^7$ cell/ml). 0.1-0.2 ml of the cell suspension was instilled into each polyvinyl alcohol sponge. Control sponges containing an equal volume of serum-free tissue culture medium only. The sponges (4 per animal) were inserted aseptically into 250 gm rats under pentobarbital anesthesia in subcutaneous pockets. The recipient rats were the same inbred Fischer strain from which the cultured fibroblasts had been derived.

In sponges removed four days after implantation, a significant increase in collagen content after inoculation with repair tissue fibroblasts was observed although even in the fibroblast-inoculated sponges the collagen content was low at this time. After seven days, the collagen content of the fibroblast-inoculated sponges was more than twice as high as in the controls. In fact, the amount of collagen present after 7 days in fibroblast-inoculated sponges was approximately the same as that in non-inoculated sponges after 14 days. The increase was about equal when skin fibroblasts and when fibroblasts from repair tissue were inoculated, or, in one experiment, from human lung (WI-38).

Preliminary experiments, in which rapidly proliferating fibroblasts were tagged by pulse-labelling and were then instilled into sponges prior to implantation into rats, showed that 10 - 15% of the radioactive label was present in the sponges at the time of removal one week later. This radioactivity was presumably present in viable cells since dead cells and cell debris would likely have been removed from the tissue within this time period. The fibroblasts in the inoculum, although only a fraction of them may remain viable, are thus sufficient to provide a significant acceleration of collagen synthesis during early stages of wound repair.

In other experiments, cultured rat fibroblasts, immobilized in plasma clots, were introduced into dorsal skin incisions in rats to determine whether wound breaking strength would be affected by the instillation into the wound of collagen synthesizing fibroblasts at the time of wounding. We believe that there was a mechanical problem in maintaining the instilled fibroblasts at the wound site, since the skin of the rat is relatively thin and it is certain that most of the instilled fibroblasts do not stay in the wound itself but are distributed in the underlying tissue. Despite this, in two of three experiments in which cultured fibroblasts derived from reparative tissue formed in implanted polyvinyl alcohol sponges were instilled into the incision at the time of wounding and immobilized in a plasma clot produced by the simultaneous instillation of a small amount of plasma and thrombin, there were statistically significant ($p < 0.01$), though modest (10-20%) increases in wound breaking strength, 12 days postoperatively. However, in only one of seven other experiments with cultured fibroblasts of other types (e.g., derived from the skin of rat embryos) was an accelerating effect on the gain of wound strength seen. These data suggest that there may be something special about fibroblasts derived from actively repairing tissues.

c. Long-term Effects of Cultured Fibroblasts, with Emphasis on Possible Tumor Formation

Background

The capacity of culture-grown cells derived from malignant tumors to cause tumors when re-injected into animals has been studied by a number of investigators. Southam, Moore and Rhoads (3) established that human established cell lines originating from malignant tumors survived in terminal cancer patients, forming nodules which persisted up to several weeks in cancer patients but for much shorter periods in normal subjects. Similar results were reported by Nadler and Moore (10) who also injected human malignant tumor cells into cancer patients and found local persistence of the cells for several weeks but no tumor propagation and no systemic reaction.

Earle and his associates (18) (19) studied by in vitro cultures and injection technique possible changes in the morphology of fibroblasts derived from normal mice and their ability to lead to tumor formation when injected (after culture) into mice, particularly when the cells were cultured in the presence of a carcinogen (20-methylcholanthrene). They presented in 1943 the results of injection of seven strains of fibroblast cultures into young C3H mice; these strains had all been derived originally from a primary culture of fibroblasts of a C3H mouse. Included were two control strains (i.e., no methylcholanthrene added knowingly to the cultures), one of which was altered very slightly morphologically during successive passages in culture while the other was more altered. The cells grown in the presence of methylcholanthrene showed substantial morphologic changes. Cultures of each cell strain on reinjection into C3H mice gave rise to tumors. The alterations in the control cultures were thought by Earle and associates to have probably having resulted from trace contamination of the control cultures with 20-methylchloranthrene, although the possibility of the changes having arisen spontaneously could not be ruled out. These various strains of cells were kept growing by Earle and associates for many years, but the methylcholanthrene was removed from the culture media. As the years went by, those cultures of cells originally treated with methylchloranthrene led to fewer tumors

when injected into mice, but the control strain which had led to the highest incidence of tumors when injected continued to maintain this property (20).

Aaronson and Todaro (11) observed that cells of established mouse embryo lines formed tumors when reinjected in mice. The cell lines used were all derived from a single pool of 14 to 17 day old Balb/c mouse embryos. "Balb/c mouse embryo lines maintained in culture for over 200 generations under conditions that minimize cell-cell contact do not become tumorigenic but lines cultivated under conditions where there is extensive cell-cell contact become tumor-producing within 30 generations. The tissue-culture property that correlates best with tumorigenicity is the loss of contact inhibitions of cell divisions".

Southam, Moore and Rhoads (3) stated that normal diploid human embryonic fibroblasts did not produce tumors in any of their experiments, and indeed all the cell lines that were found to produce tumors in the above experiments were heteroploid.

These findings were confirmed more recently by Petricciani et al. (12) who tested the tumorigenicity of a large number of cell lines in primates treated with antithymocytic globulin. In these animals, whose immune rejection system was inhibited, none of the non-transformed diploid cell lines, or primary cell lines, or primary cell explants, including WI38, chick embryo and duck embryo fibroblasts produced tumors, while established cell lines or virus-transformed cells were almost 100% tumorigenic.

Our long-term experiments - Long-term studies were carried out in which the possible causation of tumors by the re-implanted fibroblasts was monitored. Culture-grown rat fibroblasts (2-3 x 10^6 per site) were injected subcutaneously into young rats while in another group of rats polyvinyl alcohol sponges, inoculated with 2-3 x 10^6 cell each, were inserted subcutaneously; control rats for the latter received the sponges, but no cells; control rats for the former received only the media in which the cells were suspended.

In these studies, a suspension of highly inbred Fischer rat fibroblasts cells derived from the reparative tissue of polyvinyl alcohol sponge implants and grown in tissue culture (15 subcultures over a 2 month period) was added to sponge implants in other Fischer rats of the same highly-inbred strain and allowed to remain implanted for a year or more. When examined just before planting, the cultures of cells pooled appeared unchanged morphologically after 15 passages. For six months, these rats grew well and no abnormal signs were noted. Palpable tumors at the side of the sponge implants with cell suspension were found in 4 of 10 rats after seven months. Eventually all of the rats developed massive tumors on the side with the sponge implant containing added cell suspension; no tumors developed in the other side of the rats with sponge implants containing added media only.

Small pieces (1x1 mm) of viable tumor (fibrosarcoma, histologically) were excised and implanted subcutaneously in rats; all rats developed tumors by two weeks after the injection. A suspension of tumor cells was prepared by grinding pieces of viable tumor tissue suspended in Dulbecco's culture medium in a glass Potter-tissue homogenizer and the suspension injected subcutaneously in other rats. For 4-5 weeks, no palpable tumors were noted but during the next month, all (5 out of 5) rats developed tumor sites where the suspension was injected.

In other rats, a 0.22 μ Millipore filtrate of the cell suspension of viable piece of tumor tissue was injected subcutaneously. Over a 12 month period, these rats grew normally and showed no signs of tumor formation.

This study was repeated in other Fischer rats with sponge implants containing fibroblasts derived from added wound sponge fibroblasts. These cell cultures were examined just before planting and appeared morphologically unchanged after 8 subcultures. For the past five months, these rats grew normally with no untoward signs of abnormalities, but by 12 months all had developed tumors, which were readily transplanted to other rats.

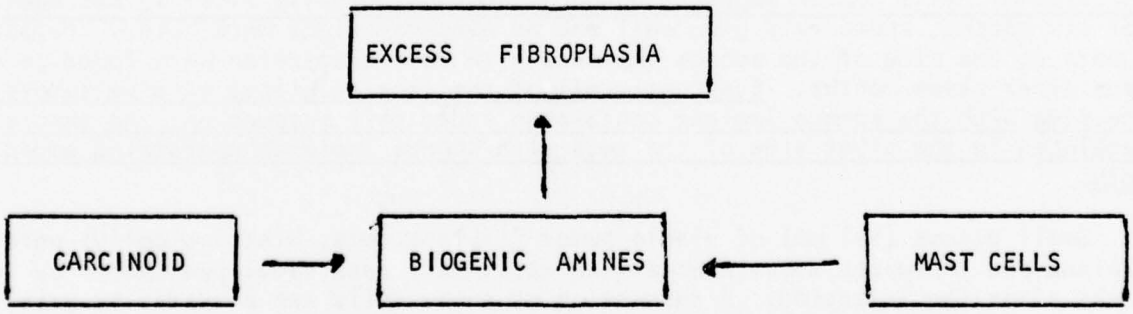
Conclusion

In view of these findings, it would appear that if one is to attempt to speed wound healing by the implantation of cultured fibroblasts, only "young" cultures must be used, before any possible transformation has occurred. These findings make us concerned about this current use by others of skin epithelial cells grown in culture for many generations and then used as skin transplants in burn patients, especially children. The possibility of late neoplasia cannot be ignored.

A.2) Studies with Compound 48/80 (21) (22)

Situations exist clinically where fibroplasia is either deficient or excessive and where control of fibroplasia would be an useful achievement (Peacock and Madden and associates (23)(24) (25) (26); Levenson and associates (27)(28). Table 1 is a simplified diagram suggesting that mast cells which synthesize and release biogenic amines are thought to act as stimuli for fibroplasia. This possibility is suggested by observations indicating an association of cells which produce biogenic amines with excessive fibrosis. For example, carcinoid tumors are commonly associated with excessive fibrosis not only at the tumor site but also at sites distant from the tumor. Carcinoid tumor cells produce and release large amounts of serotonin, a compound claimed by some to enhance fibroblastic division and collagen secretion in tissue culture. Histamine has also been implicated as having an important role in fibroplasia. On the other hand, fibrosis has been reported to follow the administration of serotonin antagonist, methysergide.

Table 1



The questions we have begun to ask are:

1. Do biogenic amines influence fibroplasia?

2. Do the mast cells have a special role in fibroplasia?

To study these questions, we chose Compound 48/80 for our initial experiments. Compound 48/80 liberates biogenic amines from mast cells by causing these cells to degranulate and discharge their amines. In rats and mice, the mast cells contain both histamine and serotonin, unlike human mast cells which contain only histamine.

In the first two experiments rats were treated with intraperitoneal injections of Compound 48/80 solubilized in 0.9% NaCl in doses of 100 μg to 150 μg for 8 days; dorsal skin incisions were made under light ether anesthesia on the 2nd day of the injection series and wound breaking strength was measured on the 7th day after infliction of the wound. In two other experiments, Compound 48/80 was given intraperitoneally on 6 consecutive days preceding wounding, in increasing doses from 100 μg to 1000 μg. In the latter two experiments there were pair-fed control groups to eliminate the potential influence of possible reduced food intake by the 48/80 treated rats. Wound breaking strength was determined on the 7th day after wounding in all experiments. Control rats in all four experiments received 0.9% saline injections intraperitoneally on the days the experimental rats received Compound 48/80 injections. In the first two experiments, wound breaking strength was decreased by treatment with Compound 48/80 which was given pre- and postoperatively. In the other two experiments, when rats received 48/80 in doses gradually increasing from 100 to 1000 μg during the 6 days preceding wounding but none in the postoperative period, wound breaking strength was significantly increased over those of the ad libitum and pair-fed control rats injected with saline. Histologic examination of the mesenteries of rats in these latter two experiments demonstrated more numerous, smaller and less densly granulated mast cells in the Compound 48/80 - treated rats. We interpret our data as indicating that the mast cells were very actively regenerating after the completion of Compound 48/80 injection series and that the wound healing taking place during this period benefited from this process.

II (2) B. Methods to Increase the Rate at Which Wounds Gain Strength by Controlling the Rate of Cross-linking of Reparative Collagen

Introduction

We pointed out in our previous research progress reports that the breaking strength of wounds is largely dependent not only on the amount of collagen present and its architectural arrangement at the gross and light microscopic levels, but also on the degree of the cross-linking at the intra- and intermolecular levels and on the associated ground substance components. Cross-linking of collagen begins when collagen is synthesized by the fibroblasts and secreted or extruded into the extracellular space and continues at a rapid rate for many weeks, following which it slows, but continues possibly throughout the liefe of the animal. The importance of the intra- and inter-molecular bonding of collagen on the strength of the collagen and thereby of the wound is illustrated by the "positive" effect of formaldehyde on the healing wound and by the "negative" effects of lathyrogens (such as B-amino propionitrile, penicillamine, penicillin, and isonicotinic acid hydrazide).

Normally, it takes a number of weeks for the major portion of cross-linking of newly formed wound collagen to occur (29) This can be accelerated in vitro in a matter of hours (e.g., by formaldehyde) (29), (Fig. 1-3), and one should be able to bring this about in vivo. How to do this, and how to limit the effects to the wound site, is the challenge, as we have pointed out in our research application and reports. Our aim is to reach a relatively "mature" degree of collagen cross-linking soon after the newly synthesized collagen is laid down in the wound without affecting collagen cross-linking elsewhere in the body (because of the postulated relationships of increasing collagen cross-linking to aging) and without interfering with the architectural rearrangement of collagen fibrils and fibers which goes on in the healing wound. We try to take advantage of the special characteristics of the healing area (e.g., the very rapid formation of collagen which is not yet cross-linked to a substantial degree) in our attempts to accomplish this.

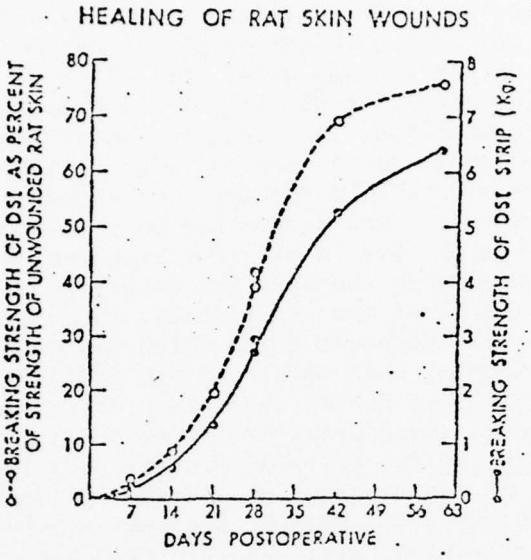


FIG. 1. Increase in breaking strength of a healing wound shown absolutely and as per cent of strength of comparable unwounded skin.

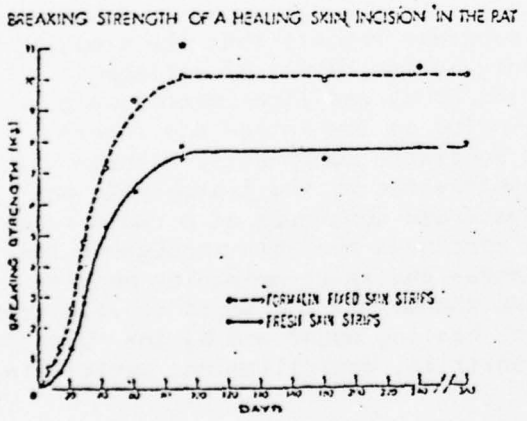


FIG. 2. Simple plot of breaking strength of a healing skin incision in rat as a function of healing time.

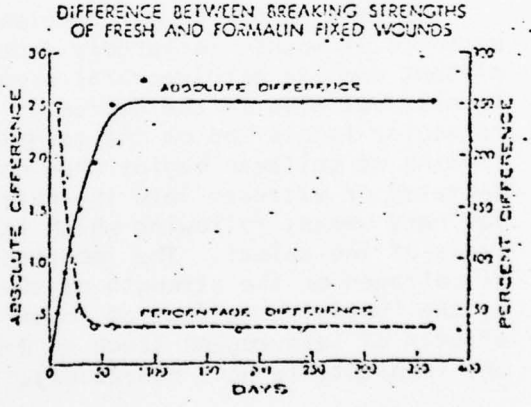


FIG. 3. Absolute and percentage differences between breaking strengths of fresh and formalin-fixed wounds as a function of time of healing.

In our specific approaches we have been concerned primarily with ways of increasing cross-linking of reparative collagen and thereby wound strength by

1. the use of enzymes to increase the rate of collagen cross-linking in the wound;

2. the use of chemicals which will cause by non-enzymatic means the aggregation and cross-linking of reparative collagen in situ.

1. To speed collagen cross-linking in situ by treatment of the wound with enzymes capable of cross-linking collagen, with the aim of improving wound strength. The enzymes to be used include diamine oxidase and lysyl oxidase.

(continued next page)

Background for use of these enzymes

The cross-linking of collagen involves the enzymatic conversion of certain E-amino groups of specific lysyl residues of collagen to the corresponding aldehydes. These aldehydes cross-link by one of two main reactions. Either they form aldimines with E-amino groups on neighboring chains or they form aldol links with other aldehydes. Tanzer has written an excellent review of collagen cross-linking(30) and a brief outline of the current status of collagen biosynthesis and cross-linking has been published by Kivirikko and Ristelli (31).

A number of years ago we undertook pilot experiments with the aim of isolating an enzyme from sponge granulomas and wound fluid which could cross-link lathyritic collagen. A crude enzyme preparation was obtained which had the properties of increasing the viscosity of collagen and of altering the gelation properties of lathyritic collagen. Our aim was to treat wounds with this enzyme for the purpose of strengthening the wound.

Subsequent to this, others have shown that cross-linking of collagen is indeed mediated by extracellular enzymes. It is well established that collagen achieves its mature form as an extracellular protein. It is also clear from the work of a number of investigators that collagen is extruded from the fibroblasts before the stage where all the lysyl residues have been oxidized to the corresponding aldeyde, and before cross-linking occurred. Thus, extracellular aldehyde poor collagen accumulates in BAPN treated rats and aldehyde-rich collagen accumulates in penicillamine treated rats. A reasonable inference, then was that the cross-linking of collagen is mediated by extracellular enzymes, and this has been demonstrated (32, 33).

Amine oxidase had earlier been implicated previously in collagen cross-linking as enzymes. Thus, based upon the correlation between the lathyrogenic action of certain compounds and their inhibition of diamine oxidase (DAO) activity, several groups, e.g., Tanzer (J. Cell Biol. 22:623, 1962) (34) Levene (J. Exp. Med. 116:119, 1962) (35), Page and Benditt (P.S.E.B.M. 124: 454, 1967) (36) and ourselves (37) had suggested that lathyrogens of the BAPN type act by inhibiting extra-cellular DAO. We had further suggested that DAO probably acts in strengthening salt insoluble as well as salt soluble collagen.

The work of Siegel and Martin (J.B.C. 245:1653, 1970) (38) and Fowler and Miller (B.B.R.C., 40:1970) (39) has made it clear that enzymes derived from normal bone and cartilage a) can oxidize selected lysyl amino groups of normal and lathyritic collagen to the aldehyde stage, b) produce an increase in the molecular weight of these collagens via a cross-linking mechanism. The enzyme is a copper protein and requires pyridoxal phosphate. It has many properties of the amine oxidases but differs from the serum amine oxidase in its substrate specificity. Thus lysyl oxidase is a special example of the class of proteins known as amine oxidases. Since 1972 (Narayanan, A.S., Siegel, R.C., and Martin, G.R., BBRC 46:745)(40) there have been few advances in the purification of lysyl oxidase; purification seems not to be an active area of lysyl oxidase research. Since our work with lysyl oxidase is of an applied nature we would consider purification of the enzyme beyond the scope of the present application.

It should be noted that in the early phase of formation of reparative collagen some of the cross-links are reversible, i.e. the Schiff's base has not yet been reduced (30).

(a) Wound Healing Experiments with DAO; Local Treatment of Wounds

Our decision to use DAO experimentally was based upon the fact that DAO resembles in some ways the enzyme system actively involved in collagen cross-linking. This does not mean that we think DAO is the natural cross-linking agent. Secondly, DAO is readily available as a commercial product. The key feature involved in the use of enzymes to speed collagen cross-linking is the fact that the process is, for the most part, an extracellular one, and the enzymes involved exist extracellularly.

Recently several wound healing experiments were conducted by us using porcine DAO. Normal Sprague-Dawley male rats weighing about 250-300 g were used. Sterile DAO solubilized in saline was instilled at the dorsal skin incision site by injection under the wound at various times postoperatively. When the wounds were examined on the tenth postoperative day, there was a significant increase in the breaking strength of the wounds receiving the DAO instillations as compared with that of controls; the DAO treated wounds were twice as strong as those of the controls, $p < 0.001$, when tested in the fresh state. Strong support for the view that the effect of the DAO was to increase the rate of the reparative collagen is our finding that the breaking strengths of the skin incisions after fixation in buffered formalin were the same in both the DAO-treated and control rats. The ratio of the breaking strength of the wounds after formalin fixation to the breaking strength of the wounds in the fresh state was much greater in the DAO treated rats. The results of a representative experiment are given in the following table 1.

(continued next page)

EFFECT OF DIAMINE OXIDASE ON 8 DAY WOUNDS IN S-D RATS

GROUPS	NO. RATS	BODY WT. AT OP.,g,	BODY WT. AT SAC.,g,	FRESH BREAKING STRENGTH, g,	FORMALIN FIXED BREAKING STRENGTH,g,	FIXED B.S. / FRESH B.S.
A - NO INJECTION	8	209 ± 9	273 ± 5	221 ± 30	1032 ± 91	4.8 ± 0.8
B - SALINE INJECTION *	8	219 ± 3	287 ± 5	193 ± 13	931 ± 50	4.8 ± 0.5
C - DAO INJECTION *	8	208 ± 2	272 ± 4	344 ± 8	976 ±109	2.8 ± 0.1
F (ANALYSIS OF VARIANCE)		N.S.	N.S.	<0.001	N.S.	<0.001
LSD (FISHER LEAST SIGNIFICANT DIFFERENCE TEST)	A vs B	N.S.	N.S.	N.S.	N.S.	N.S.
	A vs C	N.S.	N.S.	< 0.001	N.S.	< 0.001
	B vs C	N.S.	N.S.	< 0.001	N.S.	< 0.001

* DAO or saline injected into wound site on days postoperatively.

Wound Healing Experiments with Lysyl Oxidase

We have prepared sizeable batches of lysyl oxidase from chick embryo connective tissue using the method of Marayanan, Siegel, and Martin (40). The method of Pinnell and Martin (56) was used to assay the lysyl oxidase activity. A series of wound healing experiments in rats similar to those just described for DAO will be carried out. We expect that these experiments will support our hypothesis, namely, that the injection of lysyl oxidase into the healing wound will speed up the cross-linking of the reparative collagen.

(continued on next page)

(c) Microcrystalline Collagen Hemostat (MCCH) and Wound Healing

At times following trauma and also during the excision of deep burns bleeding is excessive and difficult to control.

A new hemostatic agent, Microcrystalline Collagen Hemostat (MCCH) was undergoing preclinical and clinical evaluation by numerous investigators when we started our studies. MCCH (Avitene 1000 (Avicon, Forth Worth, Texas) is purified bovine dermal collagen, shredded into fibrils and converted into an insoluble partial hydrochloric acid salt. The size of the mechanically shredded fibril fragments corresponds to natural aggregates of tropocollagen, that is, they are submicron in size. This fibrillar structure differentiates it from Gelfoam, a gelatin preparation. This new agent has a potent hemostatic action and its general use in clinical surgery was anticipated (45 -- 55). Therefore, it seemed important to us to study its behavior, including effects other than hemostatic. We chose in these initial studies to investigate its effects on wound healing in animals and in man and conducted the following studies:

A. Influence of MCCH on the closure of open skin wounds in mice.

B. Influence of MCCH on the healing of skin incisions in rats.

C. Influence of MCCH on the take of split-thickness skin grafts in pigs.

D. Influence of MCCH on the healing of donor sites and the take of split-thickness skin grafts in man.

A. A full-thickness dorsal skin defect was made in mice and standard amounts of MCCH applied immediately to the wound in half the mice. There were no significant differences in wound closure rates between control and MCCH treated mice. B. A standard dorsal skin incision was made in rats. In half the rats, MCCH was put into the incision just before suturing with fine stainless steel sutures. There were no statistically significant differences in breaking strengths between control and MCCH treated rats when tested on the 8th, 20th and 40th days postoperatively. Histologic examination of the wounds showed mild inflammatory reaction surrounding the MCCH-fiber fragments, but no giant cells. Small amounts of MCCH were demonstrable at 40 days. C. Full thickness skin burns in pigs were excised one day after burning. MCCH was applied immediately in some pigs; excess MCCH was removed by saline irrigation. Split thickness skin autografts were applied. MCCH was applied to donor sites. The "takes" of the grafts were excellent and not affected by the use of MCCH and the donor sites healed uneventfully D. MCCH was used in four patients with burns, three of whom underwent early excision of full thickness skin burns and immediate autografting. The fourth underwent skin grafting to the granulating areas 3 months after injury. The MCCH was applied to some donor sites and to some areas of excision. In one patient with severe burns, wound sepsis developed equally in areas with and without MCCH and the grafts were lost. In the other three patients, there were excellent takes of the grafts at all sites. All donor sites, treated and untreated, healed normally in all patients.

III. Chemical Accelerators of Wound Healing and Regeneration Present in Wounds and/or the Blood

(1) LIVER REGENERATION

Introduction

The ability of the liver to regenerate rapidly after partial hepatectomy is striking. Thus, after two-thirds of the liver is resected in the rat, the liver remnant reaches the preoperative size of the organ within 2 weeks and then stops. A similar, but substantially slower, process occurs in man following partial hepatectomy because of severe injury to the liver (most often of the right lobe), an important form of war (and civilian) injury, or because of limited hepatic neoplasms. Conceptually, the process from some points of view, is similar to that of a healing wound: (1) What initiates the healing and regenerative processes? (2) What keeps them going? (3) What stops them?

We have been interested in the problem of liver regeneration in relation to wound healing and injury for some time. While at the Walter Reed Army Research Institute, we found that while the healing of skin incisions was impaired in rats with severe burns, liver regeneration following partial hepatectomy was accelerated in such rats. These physiologic data correlated with studies of protein synthesis and breakdown which we conducted using N^{15} glycine in these animals (1). A more detailed discussion of these aspects was given in a previous application. During the past 3 years, we have carried out studies with the following objectives:

a. Objectives

1. to study factors involved in liver regeneration;

2. to determine whether one or more blood-borne factors are present which: a). stimulate liver regeneration and/or
b). inhibit liver regeneration;

3. to attempt to isolate and chemically characterize such a blood-borne factor(s).

b. Background

The thought that the sera of animals following partial hepatectomy contain factors influencing liver regeneration in primary and secondary ways has been held for a long time. Experiments using tissue culture techniques to compare the effects of sera obtained from animals before and after partial hepatectomy have shown that fibroblastic outgrowth from liver explants and the duration of survival of cultured fibroblasts were greater when the culture medium contained serum from partially hepatectomized rats rather than serum from normal rats (Glinos and Gey, 1952) (2). These experiments followed those of Bucher and associates (1951) which showed that when one member of a pair of parabiotic rats underwent partial hepatectomy, the unoperated partner developed an increased hepatocyte mitotic rate (3).

There is considerable interest in the possible effects of insulin and glucagon on liver regeneration. These studies have derived in part from studies of the effects of the blood supply to the liver (portal vein and

hepatic artery) on liver regeneration. Thus, investigations of alterations of blood supply to the liver by shunting the portal vein or by ligating the hepatic artery have been made to determine their effects on liver regeneration. Ligation of a branch of the portal vein results in atrophy of the liver lobe supplied by that branch and hyperplasia of the other lobes (Rous and Larimore, 1920) (4). However, neither the portal blood supply nor the hepatic artery are essential for the occurrence of liver regeneration (5) (Weinbren, 1959); Weinbren has demonstrated that when a liver lobe with deficient portal blood flow regenerating after partial hepatectomy is compared with the corresponding lobe deprived of portal blood in another group of animals with intact livers, striking increases in weight, cellular and lobular size, and incidence of mitotic figures occur in the regenerating lobe. Thus, deprivation of portal blood does not prevent regeneration after partial hepatectomy. However, the portal blood supply may alter the rate of liver regeneration

Starzl and his associates (1973-1975) have carried out recently a series of imaginative experiments using the split-liver technique. In one model, (Model 1) the portal branch serving one lobe of the liver is interrupted and the lobe is supplied by systemic blood by anastomosis to the inferior vena cava. The other lobe continues to be supplied by the portal vein. Both lobes continue to receive systemic blood from the hepatic artery. In another model, (Model 2) intestinal venous blood is shunted entirely to one side of the liver while pancreatico-duodenal-splenic blood goes to the other side (6) (7).

In the first model, the liver cells on the side receiving pancreatico-duodenal-splenic blood are larger, have higher levels of glycogen and glucokinase and lower concentrations of cyclic AMP and of active phosphorylase than those on the side receiving only systemic blood. In experiments conducted using the second model, Starzl and associates found that the hepatocytes of the side of the liver receiving the pancreatico-duodenal-splenic blood were larger than those receiving mesenteric blood, though the latter were larger than those on the side of the liver in the first model receiving blood from the inferior vena cava.

Starzl and his colleagues have recently (7) reported an extensive series of experiments using their split-liver technique in normal, alloxan-induced diabetic, and total pancreatectomized diabetic dogs. The latter two groups of dogs were treated with maintenance doses of subcutaneously administered insulin during the postoperative period.

In the pancreatectomized-insulin maintained group the liver (split-liver technique Model 1) was abnormal (fat-laden hepatocytosis) especially the side supplied by the inferior vena cava. In the alloxan-treated group, the liver abnormalities were less. The rate of uptake of ^{3}H-thymidine into liver DNA following partial hepatectomy was somewhat higher by the side receiving the splenic blood, especially when the partial hepatectomy involved two-thirds of the liver. Using Model 2 of their split-liver technique they found that the dominance (in terms of size, cell division, etc.) of the right lobe of the liver (when it received hormone-rich pancreatico-gastroduodeno-splenic venous blood) over the left lobe of the liver (which received the nutrient-rich venous return from the intestines) was now almost completely eliminated and, in fact, the most active cell division was in the left lobe. In a modification

of the split-liver experimental design, when blood from the gut, pancreas, gastroduodenum, and spleen was diverted to the right lobe and blood from the hindquarters, kidney and adrenals into the left lobe, there was an especially striking hypertrophy of the right lobe and morphologic damage to the left lobe. In such dogs total pancreatectomy or alloxan-induced diabetes (with the dogs in each case receiving subcutaneous insulin) reduced, but did not eliminate, the right lobe hypertrophy, and there was not a shift of greater cell division to the left side.

Starzl and his associates state: "These findings are consistent with our earlier multifactorial hypothesis which holds that portal hepatotropic factors are mainly interreacting hormones generated by splanchnic organs and delivered straight to the liver and that the hormone interrelationships might have augumented significance because of the high concentration of nutritional substrate in the same venous blood. The observations also substantiate by direct testing the suggestion that insulin is the most important hepatotrophic factor and that it profoundly affects many aspects of liver cell structure, division, and function." (7).

In a subsequent publication, Starzl's group reported additional experiments supporting their view; they found that the infusion of insulin into the venous supply of the left liver lobe deprived of portal blood (complete portal caval shunt) prevented the atrophy which normally occurred, while the right lobe which was not infused with insulin atrophied. Glucagon alone had no effect. Glucagon in small doses did not potentiate the insulin and glucagon in large doses may have inhibited the insulin effect (8).

A number of other investigators, including Orloff and associates (9)(10)(11)(12) and Fisher (13) have carriedout experiments in which various abdominal viscera have been removed and the effects on liver regeneration noted. The results are conflicting; some experiments suggest that the pancreas is a key organ (e.g., Orloff et al.), others that the intestinal tract is involved (e.g., Fisher and associates) (13), Sakai et al. (14), Chandler (15) (See later discussion also.)

Other investigators have suggested that glucagon is an important factor in the regulation of liver regeneration (Whittemore, et al., (16), Price, et al., (17)); they showed (as had others) that liver regeneration following partial hepatectomy occurs in the absence of portal factors, with the liver remnant supplied solely by the hepatic artery. Price and his colleagues interpret this (again, as have others) that factors of portal organs modify, but do not initiate, the regenerative response to partial hepatectomy. The rats were maintained by i.v. infusions including enough insulin to maintain minimal glycosuria. Although liver regeneration following partial hepatectomy occurred, there was a 12-hour lag in their peak liver DNA response. Infusion of glucagon into these eviscerated partially hepatectomized animals lessened the lag period and restored the peak DNA response close to that of controls. This suggested to them that glucagon is one of the major factors which regulates liver regeneration.

Bucher who has been a leading investigator in the field of liver regeneration for many years has suggested that insulin, glucagon and amino acids have permissive and modulating roles, rather than prime regulating roles. Thus, in some experiments carried out by Bucher and Swaffield (18, 18a) liver regeneration following partial hepatectomy occurred in rats whose stomachs, intestines,

spleen and pancreas were removed, the rats being maintained by continuous i.v. infusions of fluids containing only glucose and electrolytes. The growth response (H^3 thymidine uptake into DNA, mitosis) was considerably slower than when portal blood was provided. When insulin was added to the infusion, insulin dosage and uptake of thymidine into DNA did not correlate. She also found that insulin did not activate DNA synthesis in eviscerated rats with intact livers. In later experiments, Bucher and Swaffield found in rats (19) eviscerated of portal splenchnic organs and deprived of a portal blood supply that:

> "Addition of insulin (with glucose) or glucagon (with FreAmine) had little effect when given separately. In combination, however, the two hormones were capable of fully restoring the DNA biosynthetic rate to normal, even when the start of the hormone treatment was delayed for 6-7 hours after the partial hepatectomy.'
>
> "The modest but significant regenerative activity inducible in the near total absence of pancreatic hormones, and the non-deleterious effect of delaying insulin and glucagon treatment for 6-7 hours in eviscerated rats, both suggest that, despite the profound influence of these hormones, additional hormonal or humoral agents may be involved in the initial activation of hepatic regeneration. The precipitous fall in portal vein insulin levels to near zero that we observed during the first several hours of regeneration in normal (non-eviscerated) rats further supports this view, as does the preliminary finding that infusion of insulin and glucagon into eviscerated but non-hepatectomized control rats failed even minimally to excite DNA synthesis in intact livers.'
>
> "Thus, although there is a likelihood that other agents may serve to initiate and even to sustain regeneration, the pancreatic hormones, acting in synergy, appear to be the major regulators of the rate and possibly also of the magnitude of the proliferative process."

Leffert (20)conducting experiments with primary monolayer cultures of fetal rat hepatocytes found that insulin and somatomedin stimulated DNA synthesis. Neither growth hormone, nor hydroxycortisone, nor $3^1 : 5^1$ GMP initiated DNA synthesis, but when added to insulin, the insulin stimulatory effect was increased. Glucagon inhibited the insulin stimulated response without reducing the basal DNA synthesis rate. Leffert (21) also has found a decrease in glucagon binding capacity by plasma membranes of regenerating liver tissue.

Gerschenson and associates (22) found that insulin had a growth-promoting effect (including the formation of polyribosomes) on epithelial cells derived from adult liver growing in culture; in the absence of insulin in the basal culture medium used, the cells did not grow unless serum was added to the medium; while serum was not required if insulin was added to the basal culture medium.

Orloff and his associates have reported a fall in portal vein insulin following partial hepatectomy (70%) of rats (23); this together with some of their other studies indicated to them that insulin does not play a primary role in inducing liver regeneration.

Morley et al. (24) found an eleven fold increase in peripheral serum glucagon 6 hours following partial hepatectomy (70%) in rats, but not after sham-hepatectomy. Serum insulin levels were unchanged compared with sham controls for 72 hours after operation. Growth hormone fell to low levels at 6 and 48 hours after the partial hepatectomy; total serum thyroxine and free thyroxine levels also fell at 24 - 72 hours. It should be noted that sham-hepatectomy was performed and then 6 weeks later the partial hepatectomy performed in the same rats. They concluded that neither growth hormone, thyroxine nor insulin are primary stimulants of hepatic regeneration but that glucagon may modify hepatic regeneration.

Whittemore, Voorhees, and Price (25) reported additional experiments in rats with total biliary diversion and removal of the entire gastro intestinal tract, spleen and pancreas and partial hepatectomy (68%). The rats were given glucose, electrolytes and water intravenously. The blood supply to the liver was varied: hepatic artery alone, hepatic artery plus arterialized blood (from mesenteric artery) through the portal vein. Insulin was given at low and high levels and glucagon was given at varying amounts to both insulin groups. Liver regeneration was assessed principally by uptake of ^{3}H-thymidine into hepatic DNA and measurement of total DNA, RNA, and protein. They interpret their study as showing "(1) glucagon balances the effect of insulin to produce maximal response to partial hepatectomy in eviscerated rats regardless of the hepatic blood supply. (2) Augmentation of hepatic blood flow in eviscerated rats increases the DNA synthetic response to partial hepatectomy and increases the sensitivity to glucagon in balancing the effects of insulin." It should be noted that in all experiments the blood going to the liver was arterialized.

Duguay and Orloff (26) have just recently reported a study of liver regeneration following 70% partial hepatectomy in dogs with and without total pancreatectomy and with and without insulin replacement to maintain normal peripheral blood sugar levels. The dogs received no food by mouth for 1 day before and for the 2-3 days postoperatively. Five percent glucose in saline was injected continuously during the postoperative period. The pancreatectomy when done was done at the same time as the partial hepatectomy. Using the uptake of ^{3}H thymidine into DNA (assayed chemically and radiautographically) and mitotic counts as indices of regeneration, they found marked depression of regeneration in the pancreatectomized dogs not receiving insulin, a depression which was only moderately elevated when insulin was given. They concluded that these experiments confirmed their view that the hepatotropic factor originated in the pancreas, but that insulin is not this factor. No dogs undergoing partial hepatectomy and removal of some organ other than the pancreas was not done; this would have served as a useful control

In an accompanying report, Lee, Duguay and Orloff (27), found that perfusion of a non-regenerating liver isograft with a crude extract of dog pancreas stimulated regenerative activity as compared with perfusion with saline or no perfusion. No perfusions were done, however, with extracts of other tissues as additional controls.

Chandler (15) used rabbits because their unusually divided portal blood tributaries make possible ready separation of the pancreatic-duodenal venous blood from the rest of the sphlanchnic blood and the ready direction of "portal" blood of various combinations to the caudate lobe or the larger main liver mass. They concluded from a number of experiments that "The insulin-containing pancreatico-duodenal component of portal blood is not adequate, by itself, to prevent liver atrophy or to stimulate DNA replication selectively. The hepatotrophic activity of rabbit portal blood is either markedly volume-dependent, or primarily a property of venous blood emenating from splanchnic viscera other than the pancreas and duodenum." No rabbits with partial hepatectomy were studied.

Sakai, et al. (14) have found as have others (28) (29) and ourselves that the serum of rats following partial hepatectomy stimulates the growth of hepatocytes in culture. In subsequent tissue culture studies using primary explants of hepatocytes, sera samples of blood were sampled a) from the "common" portal vein, the "pancreatic" portal and the "mesenteric" portal veins of rats with and without 70% partial hepatectomy and b) the mesenteric vein of rats with total or partial resections of the intestine or splenectomy 1-2 months earlier. The sera samples were added to the basal culture medium and the effects on the hepatocyte multiplication noted. They concluded that in normal rats the serum hepatropic factor originated from the ileum and that an inhibitor was present in splenic vein blood. The sera hepatotropic activities were unchanged in the "common", or "mesenteric" portal blood after partial hepatectomy, while the level rose in the "pancreatic" portal. Their finding of hepatotropic activity in the portal blood of normal rats may be a reflection of the culture technique they employed - their basal media contained no other serum (such as fetal calf serum). There was no apparent correlation of hepatropic activity and insulin concentrations in the various sera tested.

As will be pointed out in the section dealing with our experiments, we interpret our data along with the results of others just reviewed as indicating that while certain hormones, especially insulin and glucagon, blood supply and diet are able to influence the rate of liver regeneration to some extent, none of these factors appears to play an obligatory role in initiating liver regeneration. The nature of the stimulus to liver regeneration remains obscure. Whatever the cause of this growth phenomenon, it must account for three features: (1) the growth is confined to the liver, (2) mitosis appear simultaneously throughout the liver, and (3) the growth is limited in extent, ceasing when previous size is attained.

A possibility exists that another type of factor(s) appears in the serum after partial hepatectomy which stimulates growth of the hepatic cells. Alternatively, a factor(s) preventing the growth of liver cells in the normal animal with an intact liver may disappear from the serum following actual hepatectomy, or, indeed, both the occurrence of stimulatory and the disappearance of inhibitory factors may occur. Both in vivo and in vitro studies (some of which have already been mentioned) have been made in an attempt to demonstrate humoral agents related to liver regeneration.

One of the first evidences suggesting the presence of a serum factor in liver regeneration was provided by experiments in parabiotic rats (Bucher, et al., 1951) (3), as mentioned earlier in this section of the application. When one member of a parabiotic pair was partially hepatectomized, the unoperated partner developed an increased hepatic mitotic rate. A particularly great increase in mitotic rate in the unoperated animal occurred in triplet parabiotic rats in which two animals were partially hepatectomized. While some subsequent observations have confirmed this report (Weinbren, 1959) (5), a recent investigation failed to do so (Rogers, et al., 1961) (30).

Further evidence in support of humoral control is derived, however, from studies of carotid to jugular cross-circulation of blood between partially hepatectomized and normal rats via polyethylene cannulas. At a rate of flow of about 2 ml/min, DNA synthesis is stimulated in the normal partner, when measured 20 hours after the beginning of the exchange; if the exchange lasts less than 10-12 hours, the expected DNA rise after 20 hours does not occur (Moolten and Bucher, 1967)(31). Sakai (32) also carried out cross-circulation experiments supporting the view of humoral control of liver growth.

Injections of serum from partially hepatectomized rats have been reported to increase the mitotic rate in livers of normal rats (Friedrich-Freksa and Zaki, 1954) (33). However, other investigators reported contradictory results (Weinbren, 1959) (5).

More consistent evidence for the existence of serum factors influencing liver growth has been obtained by measuring the effect of injections of serum on ^{32}P or ^{3}H - thymidine incorporation into liver DNA, in addition to comparing mitotic rates. It was found that the injection of serum from normal animals greatly reduces DNA synthesis, whereas the opposite effect was obtained when serum drawn after partial hepatectomy was used (Smythe and Moore, 1958) (34).

Strong support for the concept of humoral control has come also from a series of experiments with hepatic autografts. Investigators have shown that bits of liver implanted in regions remote from portal blood, proliferate in response to partial ablation of the parent organ (Leong, et al., 1964) (35).

We have already mentioned that tissue culture techniques have been used to compare the effects of serum obtained from animals before and after partial hepatectomy. Fibroblastic outgrowth from liver explants and the duration of survival of cultured fibroblasts were greater when culture medium containing serum from partially hepatectomized rats was compared with medium containing serum from normal rats (Glinos and Gey, 1952) (2). Tissue culture studies will be discussed again in relation to some of our own investigations.

There have been some studies suggesting that the liver itself may produce factors which (a) stimulate, and (b) inhibit liver regeneration. A number of

isolated and cross circulated liver perfusion studies have been performed by Levi and Zeppa (36) which suggest that there is a substance in the perfusate obtained from hepatectomized rats (24 hours after operation) that causes an increase in DNA synthesis when perfused into normal rat liver. These authors interpret their data as showing that the liver remnant following partial hepatectomy is the source of the humoral factor stimulating liver regeneration. These results have not yet been confirmed, though as far as we know, only one other group of investigators have attempted to carry out experiments similar to, but not identical with, those of Levi and Zeppa. (See discussion of our studies).

Fisher (1971)(13), however, interpreted data of his experiments using extracorporeal carotid to jugular circulation that a humoral factor responsible for liver regeneration does not arise from the liver remnant. Fisher found that while intact livers of normal rats incorporated thymidine in proportion to the amount of liver removed in the cross-circulation partner, the greatest response occurred after total hepatectomy. Evidence from porta caval-shunted partially hepatectomized animals cross-circulated with normal animals indicated to Fisher that the factor is in portal blood, and that the onset of regeneration is the result of a quantitative imbalance between the available portal blood factor and the number of liver cells present. (See also preceding discussion of possible portal blood factors and later discussions of our experiments.)

The technique of plasmapheresis has been used in studies of liver regeneration. Dilution of plasma, accomplished by plasmapheresis, has been found to cause stimulation of mitosis in livers of otherwise intact rats (Glinos, 1967) (37). Conversely, concentration of plasma brought about by fluid restriction has been shown to inhibit mitosis in partially hepatectomized rats (Glinos, 1967) (37). On the basis of the above results it was speculated that an inhibitor is normally present in the serum of intact rats. The decrease in plasma albumin which occurs after partial hepatectomy has been suggested as a possible cause of regeneration (Glinos, 1958), but there is not enough evidence to support this theory (38).

Leffert (79) has reported that "dialyzed fetal bovine serum contains two growth-controlling macromolecular fractions: one stimulates and the other inhibits proliferation of primary cultured differentiated fetal rat hepatocytes." Intraperitoneal injections of Serum Fraction I as prepared by Leffert (ammonium sulfate, 50%, pH 7.4, 4°C precipitates both fractions) produced stimulation of liver mitosis, while Serum Fraction II suppressed <u>in vitro</u> incorporation of CH_3-[^{3}H] thymidine into DNA. Mixing experiments showed that SF I and SF II mutually antagonize one another. Leffert concluded that both the relative and absolute serum levels of multiple factors control the growth of fetal hepatocytes in vitro.

It is generally believed that the stimulus for hepatic regeneration is tissue specific. There is however experimental evidence suggesting that this may not be so. Cardoso and his associates (39) studied the effect of partial hepatectomy upon the circadian distribution of mitosis in the cornea of rats and found evidence of increased mitotic activity following partial hepatectomy. Paschkis nad his associates (40) reported similar findings. A stimulatory effect on tumor growth following partial hepatectomy was also observed by Trotter (41) who studied subcutaneously transplanted hepatomas in mice. Bucher (42) believes that the non-specific effects that have been reported are slight and do not challenge the notion that the hepatotropic factor acts primarily on the liver cells. Gentile, Ali and Grace (43) found that serum from rats 24 hours following partial hepatectomy, in concentrations of 0.6 to 5% stimulated the growth of

primary cultures of embryonic rat fibroblasts. (See also later discussion of our experiments). In this regard, Bucher and Malt also believe that the non-specific effects that have been reported are slight and do not challenge the notion that the hepatotropic factor acts primarily on the liver cells (44). However, Sakai, Pfefermann and Rountz (44a) have recently reported experiments indicating that serum obtained from rats subjected to 70% partial hepatectomy stimulated both hepatocytes and lymphocytes in culture. (See also discussion of our studies on specificity of hepatotropic factor(s)).

Interest in substances which may inhibit wound healing and regeneration has increased recently (Bullough, 1960)(45). It has been speculated that tissue-specific mitotic inhibitors are released in the extracellular fluid and can, by a simple negative-feedback mechanism, control the size of normal tissues in the adult organism and regulate healing and regeneration. These tissue-specific mitotic inhibitors have been named chalones. One of the best studied is the epidermal chalone; it is a protein of 30,000 to 40,000 daltons that has been purified 2,000-fold (Boldingh and Laurence, 1968)(46). Bullough, et al., (1967), have shown that although the chalone is tissue-specific, it is not species-specific (47). The chalone inhibits DNA synthesis; its action is on G_1 and G2 phases of the cell cycle (Elgjo, 1969)(48). Tissue-specific mitotic inhibitors have so far been demonstrated in about 10 tissues. Verly and his associates (1971) have purified from rabbit liver a chalone (450-fold) which they have shown is a polypeptide of low molecular weight. These authors measured chalone activity by the inhibition of DNA synthesis in liver slices from the remaining lobe of a subtotally hepatectomized rat (49). As far as we know no attempt to isolate a chalone from regenerating liver has been reported.

Recently a number of investigations dealing with polyamines following hepatectomy have been reported. Polyamines are widely occurring substances which are thought important in nucleic acid function. They are known growth factors for micro-organisms and cells in culture, and their importance in liver regeneration has been indicated (50-55). Fausto has shown (50) that ornithine decarboxylase (the rate limiting enzyme in polyamine synthesis) increases very soon after partial hepatectomy and reaches a peak at about 18 hours. The rise occurs at the same time liver RNA is rising. This very early rise in ornithine decarboxylase has led researchers to consider the enzyme as having prime significance in the regenerative process. The conversion of ornithine to the polyamine putrescine occurs at a rate 60 times normal within 8 hours of the hepatectomy (50); the concentration of putrescine doubles within 4 hours and remains elevated for several days. Spermidine accumulates more slowly and spermine even slower. Actinomycin D given at the time of partial hepatectomy prevents this acceleration. Giving Actinomycin D 30-60 minutes after the partial hepatectomy is much less effective in depressing the increase in ornithine decarboxylase. The early increase in this enzyme is also prevented by prior hypophysectomy and restored when growth hormone is given to hypophysectomized animals. In some in vitro studies with rat hepatocytes, Koch and Leffert found no special role in promoting growth polyamines when added to the basal tissue culture medium (56). Bissell (81,82) and Jeejeebhoy (83,84) have conducted many studies dealing with hepatocyte cultures.

C. Studies Carried Out by Us

As described in the preceding section there are reports to suggest that a) a factor appears in the serum of hepatectomized animals which stimulate

liver regeneration, b) a factor is present in the serum of normal rats which inhibits regeneration, and c) both a) and b) are true. Further, evidence has been obtained indicating that certain hormones (e.g., insulin, glucagon) modify the rate of liver regeneration but that liver regeneration can begin in their absence albeit at a substantially slowed rate. Our aim was to clarify some aspects of these matters.

One of the central techniques employed in our studies was to determine the effects of sera and sera ultrafiltrates from rats and humans on the growth in tissue culture of E_1 cells, hepatocytes derived from the foetal rat liver, which we obtained from Dr. Harry Eagle at the Albert Einstein College of Medicine. A review of these studies was given in our research application and the results of our experiments have been published in a number of papers (57-65). Only a brief summary is included here. We studied sera of rats, dogs, and humans under a wide variety of conditions.

We interpret our studies carried out so far as indicating the following:

Studies in Rats and Patients

1) a serum factor(s) inhibiting liver regeneration is not present in normal rats or humans;

2) a serum factor(s) stimulating liver regeneration is present following partial hepatectomy in rats and humans;

3) neither total portal vein gradual occlusion in rats, nor 99.5% pancreatectomy in rats, nor unilateral nephrectomy in rats or humans affect the appearance and activity of this serum factor(s) following partial hepatectomy;

4) this stimulating factor(s) is ultrafilterable and has a MW of 5,000 - 12,000;

5) glucagon and insulin each stimulate the growth in tissue culture of E_1 cells when added to media containing sera from normal or sham-hepatectomized rats. These effects are much less when the insulin and glucagon are added to media containing sera from rats with partial hepatectomies. It is of interest that insulin and glucagon have similar effects in this system, whereas in many physiological situations they act antagonistically;

6) although insulin and glucagon modify the rate of liver regeneration, it is unlikely in our view that they are primary initiators of this process.

7) The hepatotropic activity of sera from rats following hepatectomy as assessed by the growth of E_1 cells in culture is independent of its insulin content, since serum from rats undergoing partial hepatectomy after 99.5% pancreatectomy and maintained on small doses of oral insulin has undiminished hepatotropic activity despite very low levels of serum insulin (levels which are the same low values seen in rats with 99.5% pancreatectomy and sham-hepatectomy whose serum has no hepatotropic activity). Measurements of serum glucagon (pancreatic and gut) are under way in our laboratory in similar experimental situations;

8) the cellular and tissue specificity of the hepatotropic factor(s) is still unsettled; we found that sera from partially hepatectomized rats accelerated not only the growth of E_1 cells (hepatocytes derived from foetal rat liver) but also the growth of fibroblasts derived from rat embryomic tissue but not fibroblasts derived from the skin of adult rats;

9) the concentrations of certain amino acids may modify the rate of liver regeneration, but they are not the primary hepatotropic factor(s). Following partial hepatectomy (70%) in rats there is a prompt change in most of the serum amino acid concentrations (66). Most amino acids increase, generally by a factor of 1.5-2. Ornithine goes up 4-fold while arginine falls slightly and alanine is unchanged. We tested the effect of varying amino acid mixtures on the growth of E_1 cells in culture by adding supplements of these mixtures to the basal culture media. We found that the mixture simulating the changes in serum amino acid concentrations 4 hours after hepatectomy accelerated the growth of the E_1 cells while mixtures simulating the serum amino acids of normal rats or rats undergoing sham-hepatectomy had no such effect. Such changes in amino acid concentrations are <u>not</u> the basis for the action of the serum hepatropic factor(s) we have demonstrated, since the hepatotropic factor(s) has a molecular weight 5,000-12,000 and thus substantially larger than the free amino acids. Leffert (56) has found that arginine and ornithine are obligatory amino acids for the growth of hepatocytes in culture.

10) <u>Preliminary Studies of the Nature of the Serum Hepatotropic Factor(s) In Rats</u>

Few investigators who found a factor stimulating liver regeneration have made few attempts to isolate and chemically define this factor. Gentile and his associates (43) on the basis of enzymatic inhibition studies suggested that RNA may be the stimulating factor present in serum. Wrba and Volm (68) suggest that liver regeneration may be the result of increased catalase levels in the serum following hepatectomy.

Morley and Kingdon (69) have reported the appearance of a polypeptide(s) in serum 12-36 hours following partial hepatectomy in rats which stimulates hepatic DNA synthesis. The peptide(s) is resistant to boiling. This is not related in Morley's view to growth hormone, insulin, or thyroxin since he and his colleagues found that these hormones do not increase in serum from partially hepatectomized rats in this time postoperatively (12-36 hours). Serum glucagon levels do increase

but Morley doubts that RF_1 is glucagon since the latter is not a very effective stimulant of liver DNA synthesis.

As mentioned, the results of our initial fractionation experiments show clearly that the stimulating factor present in serum following partial hepatectomy is ultrafiltrable and has a MW of 5,000-12,000. Studies are under way to elucidate the chemical nature of this factor(s) These studies involve fractionation using Sephadex columns and chromatographic techniques. Attempts are also being made to determine whether the in vitro cell stimulating activity following partial hepatectomy is also present in the urine of the hepatectomized animals in the early postoperative period. Preliminary experiments involving enzymatic screening of the serum activity showed that the in vitro cell growth stimulating activity is not lost following incubation in the presence of RNase, DNase, and Trypsin.

Holley, Kierman and associates (70-73) believe that there are four factors found in normal serum which can initiate DNA synthesis in 3T3 cells (3T3-4A cell line, a clone of Swiss mouse embryo fibroblast cell line). Three of these factors can be replaced by known substances, e.g., insulin, dexamethasone, and the fibroblast growth factor (a polypeptide, 13,300 MW as purified from bovine pituitary) of Gospodarowicz (1974)(74). It is possible that the activity of some or all of these factors is increased following partial hepatectomy in addition to the appearance of specific hepatotropic factor(s).

Pickart and Thaler (75) have described a tripeptide in the serum of normal human subjects which stimulated macromolecular synthesis (but not growth) and prolonged the survival of normal rat hepatocytes, maintained in monolayer culture and accelerated DNA, RNA, and protein synthesis and cell growth of protein synthesis and cell growth of neoplastic cells derived from Morris rat hepatoma 1239C (HTC cells). The tripeptide is bound to albumin and α-globulin in human serum, from which it could be extracted with heat denaturation of the protein fraction. The activity of the trypeptide is potentiated by serum. Chemically synthesized Gly-Lys-His or Gly-His-Lys were effective substitutes for the native factor.

Studies in Dogs

11) Sera from dogs, normal, sham-hepatectomized, and partially-hepatectomized (30% and 60%) behave in ways comparable to those of parallel groups of rats, that is, we found no evidence of stimulator(s) or inhibitor(s) in the sera of normal or sham-hepatectomized dogs, but we did find evidence of stimulator(s) in the sera of dogs obtained 7-14 days following partial hepatectomy , as assessed by the growth of E_1 in culture.

12) We had previously reported on experiments in which rats were subjected to 99.5% pancreatectomy and found that the sera of such rats did not stimulate the growth of E_1 cells in culture, but the sera of such rats which had a subsequent partial hepatectomy (70%) did stimulate the growth of E_1 cells, and to the same extent as rats with intact pancreases subjected to partial hepatectomy. However, 99.5% surgical pancreatectomy in rats is a very difficult technical procedure because of the nature of the blood supply to the duodenum and some of the rats operated upon may develop ischemic necrosis of the duodenum, a factor which complicates such a study. By contrast, total pancreatectomy in the dog is a technically simple procedure. Further, cannulation of various vessels for acute, sub-acute, and chronic experiments is much easier in the dog than in the rat. Also, there are considerable data in the literature regarding liver regeneration in the dog, including the ingenious experiments of Starzl and his colleagues.

In our initial studies, we found that serum of a dog subjected 4 days previously to partial hepatectomy (30%) three months following total pancreatectomy stimulated the growth of E_1 cells in culture to the same extent as sera from dogs with intact pancreases subjected to similar partial hepatectomies. The pancreatectomized dogs had been kept on insulin lente until three days prior to partial hepatectomy when the insulin was stopped. We have shown previously in this same dog that within 2 days following the last injection of the insulin lente, immunoreactive insulin was not detectable in the peripheral blood; in this experiment, the dog was without insulin for 7 days.

Measurements of serum immuno-reactive insulin and serum immunoreactive glucagon in normal dogs, sham-pancreatectomized, sham-hepatectomized pancreatectomized, and pancreatectomized-partially hepatectomized dogs were obtained in later experiments; these data are to be correlated with the effects of sera from such dogs on the growth of E_1 cells in culture. We also followed the uptake of ^{3}H-thymidine into DNA in various parts of regenerating and control livers to follow the rate of liver regeneration following partial hepatectomy in dogs subjected to sham-pancreatectomy or pancreatectomy. These data are being analyzed and will be reported in a subsequent progress report.

13. Some Ancillary Studies Concerning the Possible Source (s) and Nature of the Serum Hepatotropic Factor(s)

a) Does the regenerating liver produce an hepatotropic factor(s)?

In recent experiments (77) we used the approach of testing the effect of "conditioned" tissue culture media on the growth of hepatocytes *in vitro*. The term "conditioned" refers to media in which cells have been growing in culture and then the cells removed and the media used to assess the growth of heavily added cells.

Koch and Leffert(78,79) have found that serum-deficient conditioned serum obtained from primary cultures of fetal rat hepatocytes initiates DNA synthesis and mitosis in homologous quiescent monolayer cultures. The effect may be tissue-specific since they found that serum-deficient conditioned media from 3T3 fibroblast cultures was inactive for the fetal rat hepatocytes. Arginine and a lipid or lipid containing material were shown to be two of the active factors in the conditioned medium obtained from the fetal rat hepatocytes.

For our experiment rat hepatocytes were obtained from adult rats which had undergone no operation, sham-hepatectomy, or 70% hepatectomy 24 hours previously. The livers were perfused *in situ* according to the method of Bonney (In vitro 10:130, 1974) and monolayer cultures were established. At several time intervals the media from the normal liver cells were substituted with "conditioned media" from 70% hepatectomized, sham-hepatectomized, and other normal liver cells. Twelve-hour conditioned media from regenerating liver cells produced a three-fold stimulation of growth of normal hepatocytes on days 2 to 6. Conditioned media from normal or sham-hepatectomized liver cells had no effect on cell growth.

The activity of the enzyme ornithine decarboxylase (ODC) in the hepatocyte monolayer cultures was measured, since ODC levels are known to increase early in situations where active protein synthesis and cell growth are initiated. Ten-hour conditioned medium from regenerating liver cells, when added to normal hepatocyte cultures, produced a four-fold increase in hepatocyte ODC six hours later. A smaller ODC increase was produced by sham-hepatectomized or normal rat liver conditioned media.

These data suggest that hepatocytes from regenerating liver produce a factor(s) which stimulates normal hepatocyte growth *in vitro*.

We plan to continue these experiments with the aim of isolating and identifying the active hepatotropic factor(s) using methods described in the section of our proposed studies dealing with serum hepatotropic factor(s). Are these factors the same or are they different?

We believe this approach will prove fruitful. In this regard, the use of the conditioned media approach was used by Pohjenjelto and Raina (80) to identify a growth factor for fibroblasts produced by human fibroblasts *in vitro* as putrescine. Fausto, Brandt and Kestner (50) have expressed the view "The biochemical adaptations occurring in the liver fragment as a response to a decrease in organ mass is seen as the primary stimulus for the growth of the liver after partial hepatectomy."

b) Does the addition of insulin antibody and/or glucagon antibody modify the hepatocyte growth-promoting effect of sera from animals which have been subjected to partial hepatectomy?

We have described earlier in this section our studies which have led us to conclude that insulin and glucagon are modulating factors for liver regeneration, but not the prime initiating factors. We also reviewed much of the work of others dealing with the roles of insulin and glucagon. In a recent experiment, we tested the effects of the following sera on the growth of E_1 cells in culture: serum from normal and sham-hepatectomized rats, serum from rats subjected to 70% partial hepatectomy, and serum from rats subjected to 70% partial hepatectomy treated with insulin-antibody. The sera were obtained 24 hours postoperatively. We found that the insulin-antibody treatment of the serum from the rats subjected to 70% partial hepatectomy did not change the growth-promoting effect of untreated serum from the partially hepatectomized rats. We plan to repeat this experiment and to carry out similar experiments with glucagon antibody. In related experiments, sera will be treated with other absorbents, such as charcoal, to assess the effect of removing other hormones, in addition to insulin and glucagon, on the growth-promoting effect of sera from partially hepatectomized animals.

Our current studies are aimed also at the isolation and identification of the serum hepatotropic factor(s) that regulate liver regeneration following hepatic resection utilizing in vitro and in vivo techniques to demonstrate, isolate, and characterize such serum factor(s).

Sera obtained from partially hepatectomized, sham-hepatectomized and normal rats and dogs will be used and also from normal humans, patients undergoing partial hepatectomy, and patients undergoing abdominal operations not involving the liver. Different sera fractions will be tested in assay systems of rat hepatocyte cultures in vitro. We have found previously that the hepatotropic factor(s) is present in ultrafiltrates of the sera and appears to have a maximal molecular weight of 12,000. Similarly it was shown by membrane pore size to have a minimum molecular weight of 5,000. This suggests that the active factor(s) is not a free amino acid or other very small compound unless it is a small peptide (3-20 residues) bound ionically to a larger peptide of 5,000-12,000 M.W. The hepatotropic activity is not lost following incubation of the ultrafiltrates with RNase, DNase or trypsin.

The UV absorption spectra of the ultrafiltrates from normal rats and rats subjected to partial hepatectomy are being measured. We are looking particularly for quantitative differences in absorption of these sera ultrafiltrates at 200 mμ (peptide bonds), 260 mμ (nucleotides, etc.) and 280 mμ (peptides). We are looking also for new absorption peaks present in or peaks absent from ultrafiltrates of the rats subjected to partial hepatectomy compared with those from sham-hepatectomized and normal rats. If such differences are found, attempts to identify the compound(s) causing the change(s) the UV absorption spectrum will be also.

Our present hypothesis is that most likely the hepatotropic factor(s) is a peptide(s), but not, for the reasons we have mentioned earlier, insulin or glucagon. Our current analytic approach is based on this hypothesis. If our current studies suggest that the hepatotropic factor(s) is not peptide in nature, analytic schemes appropriate to the class of compounds indicated, e.g., nucleotides,will be followed.

(continued on next page)

IV. Adverse Effects of Severe Injury on Resistance to Infection; Some Aspects of Wound Infection and Wound Healing; Attempts to Increase Local and Systemic Resistance to Infection

Objective

To modify the local and systemic responses to microbial contamination and thereby increase local and systemic resistance to infection.

Our studies planned for this objective are directly related to certain aspects of the interrelationships between wound healing and wound infection, with specific reference to some of our already described wound healing investigations.

Background

The problem of wound infection is inextricably bound to wound healing; when wound healing is impaired, wound infection is more common; when significant wound infection is present, healing is delayed. There is considerable evidence that animals and patients with severe injury have a lowered resistance to infection, due to both local and systemic factors, just as we have shown that animals with severe injury have impaired wound healing. The local factors which underlie the decreased resistance to infection include tissue damage, impaired blood supply, presence of foreign bodies, and microbial contamination. The systemic factors include non-immulogic and immunologic mechanisms - vascular, cellular and humoral. The adequacy of medical and surgical care is clearly important also.

No attempt will be made to review in detail these matters here; they have been reviewed elsewhere by others (1) (2) (3) and ourselves (4). We will limit our discussion to the few aspects which we are investigating the roles of (1) Thymosin, (2) vitamin A, Metyrapone, and deoxycorticosterone (DOC) and (3) adjuvants in the prevention and treatment of bacterial and fungal wound and systemic infections.

There are a number of studies indicating impaired thymic function after injury. Rapid decrease in thymus size and number of certain thymocytes is one of the hallmarks of the reaction to any major stress. Lymphocyte depletion and impaired T lymphocyte numbers and function in burned patients and animals have been reported by others and from our laboratories (5)(6)(7). We and others have reported prolongation of the survival of skin homografts in animals subjected to a variety of stresses, further evidence of depressed cellular immunity (8)(9). There are considerable data indicating that the thymus and T-cells play important roles in determining host response and resistance to certain microbial infections and certain tumors. Castro and Medawer have proposed that agents that increase thymic size will increase T cell activity. These observations suggest that were one able to "protect" or "restore" thymic function following injury, the resistance of the injured individual to certain bacterial fungal and viral infections would be increased. Thymosin, vitamin A, Metyrapone, and DOC offer this potential. The latter three compounds also may influence in a salutary way macrophage and neutrophil function. The use of some adjuvants may potentiate these actions.

(1) Thymosin

Wound Healing: Infection

We have conducted a number of wound healing and wound infection studies with thymosin. Thymosin is a thymic lymphocytopoietic substance which has been isolated from calf thymus by White and Goldstein. Chemically the lymphocytopoietic activity is associated with a relatively heat-stable protein(s) free of carbohydrate, lipids, and polynucleotides with a molecular weight about 12,000. The effects of thymosin and anti-thymosin serum on lymphocytic production, transformation, and a variety of immunological reactions, specifically related to T cell functions and characteristics, including homograft acceptance and rejection, and growth of Moloney Viral Sarcoma have been extensively studied (11) (12) (13). It is thought that one way thymosin acts is on a precursor of immunologically competent T-cells by accelerating the rate at which related population of these precursor cells mature to uniform T-cell characteristics and functions. The specific molecular basis of this influence remains to be determined. "Suggestive data regarding a possible basis for the action of thymus factors on lymphoid cells have been presented by both Bach and co-workers and by Trainin and his collaborators at this conference indicating that, as in the case of many other polypeptide hormones, cyclic AMP may be a modulator in the process of T-cell maturation. In addition, the increased intra-cellular concentration of cyclic AMP produced in lymphoid cells by prostaglandin E_2 and the ability of this latter compound to enhance graft-versus-host reactivity indicate many approaches to the elucidation of the mechanism of action of thymic factors."(11). Thymosin may function to: (a) induce the appearance of specific types of T-cells, and (b) induce the reversion of aberant T-cells to more normal modes of function.

Thymosin is prepared generally from calf-thymus but has been demonstrated in humans (thymus, serum).

As prepared by Hooper, Goldstein and their colleagues (14), thymosin fraction 5 contains several heat-stable acidic proteins that range in MW from 12,000 to 14,000. It is not know whether all the lymphocytopoietic activity sides in a single molecular species (fraction 8, MW 12,000) or whether multiple thymic factors act in concert to endow the host with its normal complement of activity.

A number of other preparations of products obtained from the thymus by other investigators have been described. All of these are polypeptides, glycopeptides, or protein in nature with M.W.'s ranging from about 1,800 to about 170,000. A list of these appear in the paper by White (11). These may represent the various methods of preparation used by different investigators or the existence of more than one thymic hormone, or both.

The first administration of thymosin to a patient was to a child with markedly low T lymphocytes in the peripheral blood (15). After demonstrating an in vitro response of her lymphocytes to added thymosin, thymosin treatment of the child was begun. There was a significant increase in the number of T lymphocytes in her peripheral blood. Delayed hypersensitivity reactions to mumps and candida appeared, but in vitro response of her cells to phytohemaggluten and in mixed lymphocyte cultures were unchanged. Since then, thymosin (Fraction 5 of Hooper, Goldstein and colleagues) has been given to additional patients with a variety of primary and secondary immunodeficiency diseases and has partially reconstituted host immunologic competence in ways similar to those seen in several animal models.

(a) Wound Healing

The observation of Dougherty of Utah that the administration of thymosin to mice increases the number of fibroblasts in the loose areolar tissue suggested to us that thymosin might have an accelerating effect on wound healing.

A series of in vitro and in vivo experiments to establish the effects of thymosin on fibroblastic proliferation and wound healing was carried out in collaboration with White and Goldstein using a preparation of thymosin as prepared by an earlier method of theirs.

Parallel groups of mice with and without thymosin injections were studied; the thymosin was dissolved in 0.9% NaCL; the controls were injected with the saline alone. Mice were used rather than rats because mice are more responsive to thymosin. Standard skin incisions and polyvinyl alcohol sponge implants (subcutaneous) were made; body weights, food and water intakes were followed. At varying time postoperatively (2-20 days) the animals were killed.

There was a significant systemic thymosin effect in terms of increase in lymph node size (axillary,inguinal), rate of incorporation of ^{3}H-thymidine into lymph node DNA, and number of peripheral lymphocytes. There was also a significant increase in the thymosin treated animals in the number of inflammatory cells in the reparative tissue in both the skin incisions and sponge granulomas in the first week postoperatively as judged histologically, by DNA contents, and uptake of ^{3}H-thymidine into DNA, as compared with control mice receiving saline, the vehicle in which the thymosin was dissolved. Later, after the inflammatory phase passed, there was little difference between the thymosin treated and untreated mice in the healing of skin incisions assessed by the histologic appearances and the breaking strength of the wounds, and the histologic appearances and hydroxyproline contents of the sponge reparative tissues 7-20 days postoperatively.

Thymosin, thus, induced an increase in the inflammatory reaction to wounding.

(b) Infection

We have also reported previously some experiments conducted with the same thymosin preparation aimed at determining the possible effects of thymosin administration on altering the response of animals to wound infection. Morris and Burke (16) have shown that the extent of the tissue response to the intradermal injection of pathogenic bacteria, e.g., Staphylococcus aureus, is diminished in guinea pigs treated with ALS. This correlated with a decrease in the number of circulating lymphocytes. The view has been advanced that the local tissue response to such bacterial infections consists of at least two phases, direct injury by the bacteria and a cell-mediated immunologic response of the delayed hypersensitivity type. What influence this may have on the ability of the animal to resist extensive bacterial contamination, especially injured animals, was not explored in Morris and Burke's experiments.

(1) Thymosin

In our initial experiments, the burned tails of mice were dipped into a culture of Pseudomonas aeruginosa highly virulent for mice, a technique described by Rosenthal (17). Some mice received thymosin daily beginning at the time of challenge,some received the saline vehicle, some did not. The mortality in several replicate experiments was prompt, high, and similar in both groups. This is a model in which the burn injury induces decrease in local tissue resistance but little if any decrease in systemic resistance.

In another early experiment, we looked at the effect of thymosin administered to mice whose systemic resistance to infection was decreased by a single injection of cortisol and who were then challenged with Candida albicans, injected intravenously. We found that cortisol decreased the systemic resistance of mice to i.v. Candida albicans injection remarkably- for example with a dose of Candida albicans which led to a mortality of less than 10% in mice not receiving cortisol, mice receiving the single i.m. dose of cortisol (5 mg/20 g mouse) just before the i.v. injection of the Candida albicans had a mortality of 80-90%. Thymosin in low dosage had no influence on this mortality.

(2) Vitamin A, Metyrapone, and Deoxycorticosterone

Our previous and planned experiments dealing with the effects of vitamin A, Metyrapone and deoxycorticosterone on wound healing of injured animals have been discussed in Section II of this progress report.The close similarity of their effects on the inflammatory reaction and other steps in the healing process was pointed out along with a discussion of possible mechanisms underlying their actions. As we have already pointed out in this progress report, there has been considerable interest in recent years on the possible influence of vitamin A on the biochemical, physiologic, and immunologic responses of animals and patients to injury, malignancy, and infections. Cohen and his associates, among others, have written on these matters (18-21). It has been known for a long time that dietary vitamin A deficiency leads to a decreased resistance to infection. But what of the individual normally nourished who is suddenly subjected to a severe injury or infection? Our wound healing studies (38) and those of others dealing with serum and liver vitamin A levels (23, 24) and stress ulcers (25) indicate that there is an increased requirement for vitamin A under such circumstances. Will giving such individuals extra-vitamin A provide any benefit from the point of view of the prevention and treatment of infections? We think this a likely possibility?

Our previous studies may be briefly summarized as follows:

(1) the administration of vitamin A to normal mice increases thymic size and vitamin A given to stressed animals prevents the anti-thymic action due to stress (sharp reduction in thymus size and number of thymocytes) and prevents some of the immunosuppressant action of stress as judged by the ability of mice to resist a viral tumor (26, 27).(2) When mice are subjected to trauma they are particularly susceptible to the Moloney Sarcoma Virus; a trauma-viral interaction occurs so that while neither the trauma nor the viral infection are lethal by themselves, the combination leads to substantial mortality (50%). Supplemental vitamin A treatment lowers this mortality significantly (Levine, et al.(28).

(3) Vitamin A counteracts the immunosuppressant effects of stress (partial body casting) as judged by the time of rejection of skin grafts (9, 30). Schweizer and Bartus (31) found that vitamin A given to normal rabbits did not alter survival of skin allografts.

(4) In experiments of burn-induced or cortisone-induced immunosuppression, the following data were obtained by us in regard to lethality from purposeful contamination with Ps.aeruginosa or Candida albicans (32) (a) In the burned rat (30-35% body surface, third degree) single-dose i.v. Pseudomonas aeruginosa injection given 1 hour after burning is more lethal than single-dose topical application to the burn 1 hour after burning; dose-response mortality curves demonstrated this. Vitamin A treatment or prophylaxis did not protect burned rats against death from i.v. Ps. aeruginosa injection. Vitamin A had a modest ameliorating effect in 2 of 4 experiments in burned rats whose burns were contaminated purposefully by the topical application of Ps. aeruginosa.

(b) Cortisol (5 mg/20 g mouse, i.m., single dose) markedly enhanced the lethality of i.v. Candida albicans as already mentioned. Supplemental vitamin A prophylactically or therapeutically had little effect on this model of experimental candidiasis when the dose of Candida albicans injected, was rapidly fatal for all experimental mice. At a lower dose challenge of Candida albicans (but still eventually 100% fatal for the control mice) the following was found: 15% of the control mice died 7 - 11 days after challenge, 50% at 14 - 17 days, and 100% at 19 - 21 days. Some mice receiving supplemental vitamin A died earlier than some controls, but the final motality of the vitamin A treated rats was significantly lower (65%) than that of the control rats receiving corn oil (100%).

These findings indicate that supplemental vitamin A by itself does not antagonize adequately the immunosuppression resulting from a 30% third degree burn or administration of large amounts of cortisol. This is consistent with our data obtained in other experiments which showed that while vitamin A could antagonize low and moderate doses of cortisone in terms of its effects on thymus weight and composition, and resistance to Moloney Sarcoma virus, it could not adequately antagonize high doses of cortisone.

(5) Metyrapone (a compound that inhibits glucocorticoid biosynthesis, and leads to an accumulation of deoxycorticosterone) a) prevents the occurrence of gastroduodenal ulcers after restraint or a cortisone administration, b) prevents the changes in thymus weight and composition after stress, and c) protects mice against the Moloney Sarcoma Virus (29) and the C_3HB mammary carcinoma (33). Deoxycorticosterone administration also protects mice against these two neoplasms (33)(34).

(6) We have found also that while cortisone administration decreases thymic size, increases adrenal size, and slows skin graft rejection, administration of deoxycorticosterone does not lead to these changes. Cortisone and deoxycorticosterone also have opposing effects on the growth of tumors (both a viral (Murine Sarcoma) and non-viral (C_3HBA mammary), the healing of wounds and susceptibility to infections. This has led us to suggest that vitamin A anti-glucocorticoid action may result, in part, from a shift in the balance of adrenocorticoid secretion from gluco-to mineralo-corticoids.

(7) In a recent study, the vitamin A content of tissues from control and stressed animals were determined in rats receiving a chow diet and the same diet supplemented with 150,000 I.U. of vitamin A/kg diet. The stress consisted of a partial body cast applied to the thorax and kept there for three days. Male Sprague-Dawley rats weighing approximately 250 g were used.

Casting lowered the vitamin A content of tissues in the following way:

plasma, 17%; liver, 7%; adrenals, 18%; perirenal fat (white), 0%; thymus, 99%.

Feeding supplemental vitamin A increased vitamin A contents to greater than normal values in all tissues; tissues of non-casted animals had more vitamin A than those from casted rats. Among the organs examined, the thymus was most affected by casting stress in terms of vitamin A loss.

This supports our previous experiments and view that an important role of vitamin A is to support thymus-mediated immune reactions. This concept was supported further by our finding recently that vitamin A stimulates thymus growth in adrenalectomized mice and that vitamin A exerts its anti-tumor action in adrenalectomized as well as intact mice. Adrenalectomy (ADX) increases thymic weight but vitamin A given to ADX mice led to an even greater increase of thymus weight. Vitamin A also allowed tumor growth and decreased the early and late mortality resulting from inoculation of normal or ADX mice with Murine Sarcoma virus; this was used as one type of viral infection.

(8) In a current experiment, we are extending our earlier studies of the effect of vitamin A on the resistance of burned animals to infection and are including groups of animals given metyrapone alone or in combination with vitamin A. The hind limbs of mice were burned by dipping them into hot water to the level of the upper third of the thighs (70 °C, 3 seconds). The limbs were then dipped for a few seconds into 18 hour actively growing cultures of a strain of Pseudomonas aeruginosa virulent for mice; different groups had their limbs placed in cultures of one of three concentrations of bacteria: 5×10^3, 5×10^5 and 5×10^7. All mice were given 0.85% NaCl i.p. immediately after the burn and 5% glucose in 0.85% NaCl to drink for the first 24 hours post injury. The mice were divided into 4 groups: Group 1, controls, ate a commercial mouse chow; group 2, vitamin A supplemented, ate the chow modified by the inclusion of 200 mg of vitamin A/kg chow; group 3, metyrapone supplemented, ate the chow modified by the inclusion of 200 mg metyrapone/kg chow; group 4, ate chow supplemented by both vitamin A and metyrapone in the concentrations noted respectively for groups 2 and 3. The diets were begun 11 days prior to the leg burns and challenge with the Ps. aeruginosa and continued thereafter. All mice ate and drank ad libitum.

Among the four groups challenged with 5×10^3 Ps. aeruginosa organisms, only 1 mouse died (Groups 1 and 2); among those challenged with 5×10^5 organisms, all died. When the challenge was with 5×10^4 organisms, 71% of group 1, 78% of group 2, and 78% of group 4 died, while only 13% of group 3 (metyrapone treated) died.

This preliminary experiment suggests that the administration of metyrapone has a protective effect against Ps. aeruginosa infection. This experiment will be repeated and extended.

Some of our current studies are based on our belief that it is likely that the effects of vitamin A and thymosin on increasing the resistance of injured animals to infection will be additive and perhaps synergistic, because the mechanism of their effects on lymphocytes, particularly T lymphocytes, are different and possibly complemental. In addition, it is possible that vitamin A may also increase macrophage and neutrophil function, an additional factor in combating infection. Experiments exploring this latter possibility are in progress, e.g., testing the effects of supplemental vitamin A on the resistance of mice to injected endotoxin; resistance to endotoxin correlates with function of the RES. Depression of RES function after severe injury is common.

It is also likely that Metyrapone and or DOC given in addition to thymosin and/or vitamin A will also increase resistance to infection since the mechanisms of the Metyrapone and DOC actions are different from those of both vitamin A and thymosin. Metyrapone is a compound which decreases glucocorticoid output. The experiments with Metyrapone and DOC are designed principally to clarify mechanisms, not as potential therapy.

We believe also that the use of certain adjuvants will potentiate the same of the actions of the agents we are studying.

A series of experiments are in progress testing the various possibilities just mentioned. Goldstein, White and their associates have continued to purify thymosin and preparations are available to us which are considerably more active than those we used in the past. We will use principally fraction 5 as prepared by Hooper, Goldstein and their colleagues, the fraction that has been generally used clinically.

We are looking at the possible effects of thymosin, vitamin A, Metyrapone and DOC administration, singly and in various combinations, on the occurrence and severity of experimental infections where the underlying cause may be principally.

(a) lowering of local resistance,

(b) lowering of systemic resistance,

(c) a lowering of both local resistance and systemic resistance.

Among the microbial agents to be used are Ps. aeruginosa, Staphylococcus aureus and Candida albicans. All these are important clinically. There is evidence that the thymus and T lymphocytes play important roles in candidiasis, and it may be that thymosin will prove to be most useful in this model. We think vitamin A will be important in both bacterial and fungal infections.

(continued next page)

Adjuvants

There exist a variety of agents that alter animal or patient responses to infective microorganisms or certain tumors.

We believe that certain of these adjuvants are likely to help restore the depressed defense systems of injured animals and patients, particularly when used with some of the other compounds we have already described.

We know of no studies in which adjuvants have been used to help stimulate phagocytic or immune responses in animals or patients with traumatic injuries. Our aim is to use certain adjuvants to help increase the resistance to infection of burned or injured animals. Vitamin A has been called an adjuvant but for the purposes of this report it is considered separately by us. We propose to study the influence of Freund's Complete Adjuvant, B.C.G., C. Parvum and yeast glucan in the animal models already described in the section dealing with thymosin, vitamin A, Metyrapone, and DOC. These adjuvants will be used alone and in conjunction with vitamin A, Metyrapone or DOC.

In another series of experiments, the effects of supplemental arginine on the resistance of animals especially seriously injured animals, to infection will be assessed.

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THE BIBLIOGRAPHY IS ARRANGED IN VARIOUS SECTIONS CORRESPONDING TO THE TOPICS OF DISCUSSION IN THE TEXT. THE REFERENCES IN EACH SECTION ARE ARRANGED NUMERICALLY.

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III Chemical Acceleration of Wound Healing and Regeneration Period in Wounds and/or the Blood.

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